SYNTHESIS AND ANALYSIS OF HYPUSINE-CONTAINING DIPEPTIDES AND EUKARYOTIC TRANSLATION INITIATION FACTOR 5A PEPTIDE FRAGMENTS: ADDITIONAL TOOLS TO STUDY THE AMINO ACID HYPUSINE

By

MATTHEW J. DELLA VECCHIA

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Matthew J. Della Vecchia

This dissertation is dedicated to my beautiful wife,
Stephanie.

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LIST OF ABBREVIATIONS

ADC	arginine decarboxylase (EC 4.1.1.19)
$[\alpha]^{25}_{D}$	specific optical rotation at 25 °C for D (sodium) line
AdoMet	*S*-adenosylmethionine
AdoMetDC	*S*-adenosylmethionine decarboxylase (EC 4.1.1.50)
AIDS	acquired immunodeficiency syndrome
AIH	agmatine iminohydrolase (EC 3.5.3.12)
Al_2O_3	alumina, aluminum oxide
aq	aqueous
ARD	AIDS related diarrhea
Ar	argon
β-ALA	β-alanine
BBO	broad-band probe, NMR
BOC	*tert*-butoxycarbonyl
BOP	benzotriazol-1yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate
c	concentration (g/100 mL), for optical rotation
°C	centigrade degree; Celsius degree
C_6H_6	hexanes
CBz	carbobenzoxy
CD_3OD	deuterated methanol
CH_2Cl_2	methylene chloride
CH_3CN	acetonitrile
CH_3OH	methanol
$CHCl_3$	chloroform
CHO	chinese hamster ovary cells
CO_2	carbon dioxide
COOH	carboxylic acid functional group
CPM	counts per minute
δ (delta)	chemical shift
D_2O	deuterated water
DAO	diamine oxidase
dcAdoMet	decarboxylated S-adenosylmethionine
DFMO	α-difluoromethylornithine
DHyp	deoxyhypusine
DIEA or DIPEA	diisopropyl ethylamine
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DOHH	deoxyhypusine hydroxylase
DOHS	deoxyhypusine synthase

DPM	disintegrations per minute
e.g.	(exempli gratia) for example
eIF-5A	eukaryotic translation initiation factor 5A
EtOAc	ethyl acetate
FAD	flavin adenine dinucleotide
FMOC	9-fluorenylmethoxycarbonyl
GABA	γ-aminobutyric acid
h	hour
H_2	hydrogen
H_2O	water
HBr	hydrogen bromide
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate
HCl	hydrogen chloride
HIV	human immunodeficiency virus
HMQC	heteronuclear multiple quantum correlation spectroscopy
HOAc	acetic acid
HOBt	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
Hyp	hypusine
i.e.	(id est) that is
KCN	potassium cyanide
λ (lambda)	wavelength
LCMS	liquid chromatography mass spectrometry
LiAlH4	lithium aluminum hydride
M	molar concentration: moles per liter
MALDITOF-MS	matrix assisted laser desorptive time of flight mass spectrometry
MAO	monoamine oxidase
mCi	milliCurie
MeOH	methanol
2-ME	2-mercaptoethanol
MGBG	methylglyoxal bis(guanylhydrazone)
MHz	megahertz
min	minute
ml	milliliter (cubic centimeter)
mmol	millimole (10^{-3} moles)
mRNA	messenger ribonucleic acid
MS (HRMS)	mass spectrometry (high resolution mass spectrometry)
N	normal (equivalent per liter)
N_2	nitrogen
Na_2SO_4	sodium sulfate
NaH	sodium hydride
$NaHCO_3/Na_2CO_3$	sodium bicarbonate/sodium carbonate
$NaOCH_3$	sodium methoxide
NaOH	sodium hydroxide

NCPA	*N*-carbamoylputrescineamidase (EC 3.5.1.53)
$(NH_2)_2C(NH_2)$	guanidine
NH_3	ammonia
NH_4OH	ammonium hydroxide
NMDA	*N*-methyl-D-aspartate
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
OD	optical density (absorbance)
ODC	ornithine decarboxylase
ONSu	*N*-hydroxysuccinimide
OPA/NAC	*o*-phthaldialdehyde/*N*-acetyl-L-cysteine
PAO	polyamine oxidase (EC 1.5.3.11)
PAPT	putrescine aminopropyltransferase; spermidine synthase (EC 2.5.1.16)
Pd-C	palladium carbon
pH	acid-base scale: log of reciprocal of hydrogen ion concentration.
PLP	pyridoxal 5'-phosphate
ppm	parts per million
PtO_2	platinum dioxide
Put	putrescine; 1,4-butanediamine
PyBOP ®	benzotriazolyloxy-tris[pyrroldino]-phosphonium hexafluorophosphate
(*R*)	rectus (right) stereodescriptor
Rev	viral regulatory protein for HIV
R_f	TLC compound migration distance indicator
RRE	rev response element, arginine rich domain of rev protein
rpm	revolutions per minute
(*S*)	sinister (left) stereodescriptor
SAO	serum amine oxidase
SAPT	spermine aminopropyltransferase, spermine synthase (EC 2.5.1.22)
Spd	spermidine; N^1-(3-aminopropyl)-1,4-butanediamine
Spm	spermine; N^1, N^4-bis(3-aminopropyl)-1,4-butanediamine
SPPS	solid phase peptide synthesis
SSAT	spermidine/spermine N^1-acetyltransferase (EC 2.3.1.57)
TFA	trifluoroacetic acid
THF	tetrahydrofuran
THP	tetrahydropyran
TIS	triisopropylsilane
TLC	thin-layer chromatography
TMS	tetramethylsilane
TOCSY	total correlation spectroscopy
TRNA	transfer ribonucleic acid
TSP (NaTSP)	3-trimethylsilylpropionate, (sodium salt)
UV	ultraviolet

Abstract of Dissertation Presented to the Graduate School
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SYNTHESIS AND ANALYSIS OF HYPUSINE-CONTAINING DIPEPTIDES AND EUKARYOTIC TRANSLATION INITIATION FACTOR 5A PEPTIDE FRAGMENTS: ADDITIONAL TOOLS TO STUDY THE AMINO ACID HYPUSINE

By

Matthew J. Della Vecchia

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Major Department: Medicinal Chemistry

Hypusine is an amino acid originally isolated from the extracts of bovine brain in 1971. Currently, this amino acid has been located only on a single protein, eIF-5A. This 154 amino acid protein (human) undergoes a post-translational modification that transforms a specific lysine residue (Lys_{50}, human) to hypusine. Two enzymes, deoxyhypusine synthase and hydroxylase, and the polyamine spermidine are required for this highly conserved modification to occur. Despite its categorization as an initiation factor for protein synthesis, the exact function of eIF-5A is still unclear. However, the lysine to hypusine modification is essential for the protein's full function. Particularly interesting are recent experiments that have implicated eIF-5A's involvement in the progression of HIV. Inhibition of the hypusine modification on eIF-5A causes HIV replication to be reduced, thus providing a potential for a therapeutic outcome.

In order to understand and determine the functions of hypusine, eIF-5A, and its two reported dipeptides, α-(γ-aminobutyryl)-hypusine and α-(β-alanyl)-hypusine, it is imperative that reliable and efficient techniques be developed to synthesize and analyze these compounds. The proper tools to carry out these experiments are lacking as hypusine and deoxyhypusine reagents, hypusine dipeptides, and eIF-5A peptide fragments are not commercially available.

This dissertation describes the multi-step synthesis and analysis of hypusine dipeptides and ^{3}H-labeled hypusine as well as the incorporation of hypusine, deoxyhypusine, and ^{13}C-labeled hypusine reagents into eIF-5A peptide fragments using solid phase peptide synthetic techniques. The radiolabeled compound has enabled the development of a new tissue extraction method for the analysis of free hypusine and hypusine dipeptides from rat brain. Extracts were analyzed for the presence of these compounds via precolumn derivatization on HPLC and HPLC-MS systems. EIF-5A peptide fragments containing lysine, deoxyhypusine, hypusine, and ^{13}C-labeled hypusine at position 50 in human eIF-5A have been characterized by 1D and 2D high field homonuclear and heteronuclear NMR experiments. The data collected here provides a basis for the future analysis of hypusine and eIF-5A peptides and their interactions with other components of various viral proteins.

CHAPTER 1
INTRODUCTION

Polyamines

Polyamines are organic compounds containing two or more amine moieties separated by aliphatic carbon chains of varying length. The three most common naturally occurring polyamines are putrescine (Put; 1,4-butanediamine), spermidine (Spd; N^1-(3-aminopropyl)-1,4-butanediamine), and spermine (Spm; N^1, N^4-bis(3-aminopropyl)-1,4-butanediamine) (Fig. 1-1). These polyamines are considered ubiquitous since at least one of these compounds has been detected in almost all cells studied, both prokaryotic and eukaryotic, plant and animal (Jänne et al., 1991; Thomas & Thomas, 2001). These low molecular weight bases have been detected in the millimolar concentration range in proliferating mammalian cells (Cohen, 1971; Thomas & Thomas, 2001). Other polyamines, such as cadaverine (1,5-pentanediamine) and 1,3 propanediamine, occur in nature as well and have been identified as polyamine metabolites in human and rat urine (Muskiet et al., 1995).

Polyamine History

Polyamines are considered essential for cell growth and differentiation, and the biosynthetic pathways for the formation of the major polyamines *in vivo* are well established (Shantz & Pegg, 1999). Despite this fact, most biochemistry courses and textbooks typically offer only minor discussions on this group of compounds. Much less is said regarding their initial discovery. In actuality, there is a vast history to the study of

polyamines that dates back over three hundred years. The historical summary given below is taken from Cohen (1971).

One of the first scientists to report data related to polyamines was Antoni van Leeuwenhoek. In his letter to the Royal Society of London in 1678, Leeuwenhoek described the gradual formation of colorless crystals in samples of seminal fluid. The French chemist Nicolas Vauquelin also described a similar observation in 1791, apparently unaware of Leeuwenhoek's discovery some 113 years earlier. Nearly a century later in 1878, Schreiner reported that these crystals were a phosphate salt of an organic base. The name spermine was eventually given to this organic base in 1888 but it was not until Rosenheim synthesized spermine and the related polyamine, spermidine, that the correct structure of this compound was finalized in 1926 (Cohen, 1971).

The discovery of putrescine is attributed to Brieger who isolated this compound from animal tissues in 1885. Ladenburg synthesized putrescine a year later, confirmed its structure, and verified Brieger's work (Cohen, 1971). In the years that followed, more reliable syntheses of the polyamines were developed and studies to determine the exact biological functions and roles of these compounds began. A majority of the research to date has focused on the polyamines as they occur in cells of bacteria, yeast, and mammals. More recently, the role of polyamines in cells of higher plants has also been investigated (Bagni & Tassoni, 2001).

Putrescine, spermidine, and spermine are di-, tri-, and tetraamines respectively. The nature of these compounds, multiple amines separated by carbon backbones, has led to an abbreviated method to denote their structure. The number of carbons between each amine is used to describe each compound. Thus, putrescine can be abbreviated as "4,"

while spermidine and spermine are denoted as "3-4" and "3-4-3" respectively (Fig. 1-1). At physiological pH the polyamines are protonated making the compounds di-, tri-, and tetracations respectively as well. This unique polyionic nature is thought to be a main reason why polyamines tend to exhibit a wide range of effects in nature (Jänne et al., 1991). Since the 1970's, numerous studies have been published regarding the functions and roles of these organic compounds. Still it is generally considered that their exact functions and roles are yet unknown. Future study in this field is indeed warranted as the research completed to date has shown promise in terms of the potential development of treatments for a variety of debilitating human diseases such as cancer (Marton & Pegg, 1995).

Polyamine Biosynthesis

As stated, the polyamines are necessary for eukaryotic cell growth. Severe depletion of these compounds reduces growth in mammalian cells (Tome & Gerner, 1997). On the other hand, elevated polyamine levels within a cell can be toxic (Marton & Pegg, 1995; Thomas & Thomas, 2001). Cells maintain a balance among the three major polyamines and interconversion can occur in order to maintain this balance. Thus, eukaryotic cells possess a multilevel polyamine regulation system by which the synthesis, catabolism, uptake and excretion of putrescine, spermidine, and spermine can be controlled (Pegg et al., 1995).

The primary carbon and nitrogen sources for putrescine, spermidine, and spermine are the amino acids L-methionine, L-arginine, and L-ornithine (Jänne et al., 1991). Several key enzymes work in conjunction with these amino acids to create a polyamine biosynthetic pathway responsible for the production of polyamines within a

cell. These enzymes are (1) arginase (EC 3.5.3.1), (2) ornithine decarboxylase (ODC, EC 4.1.1.17), (3) arginine decarboxylase (ADC, EC 4.1.1.19), (4) agmatinase (EC 3.5.3.11), (5) agmatine iminohydrolase (AIH, EC 3.5.3.12), (6) *N*-carbamoylputrescine amidase (NCPA, EC 3.5.1.53), (7) S-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50), (8) spermidine synthase (PAPT, putrescine aminopropyltransferase, EC 2.5.1.16), (9) spermine synthase (SAPT, spermidine aminopropyltransferase, EC 2.5.1.22), (10) spermidine/spermine N^1-acetyltransferase (SSAT, EC 2.3.1.57), and (11) polyamine oxidase (PAO, EC 1.5.3.11) (Morgan, 1998; Bagni & Tassoni, 2001). Though each of these enzymes is important for the overall synthesis of polyamines, ornithine decarboxylase, S-adenosylmethionine decarboxylase, spermidine synthase, spermine synthase, and spermidine/spermine N^1-acetyltransferase are considered key. A generalized pathway for the biosynthesis of polyamines is shown in Figures 1-2 and 1-3a,b (Marton & Pegg, 1995). The pathways for the synthesis of each polyamine and the enzymes that promote these syntheses are discussed below.

Formation of Putrescine

In eukaryotes, ornithine decarboxylase (ODC) forms putrescine directly by removing the –COOH functional group from L-ornithine, releasing CO_2 (Fig. 1-2). ODC is a unique enzyme in that compared to most mammalian enzymes whose half-lives are measured in days, it has a relatively short half-life of 10 to 60 minutes depending on species (Russell & Snyder, 1969; Morgan, 1998). ODC, regardless of source, is a dimer of identical subunits and absolutely requires pyridoxal 5'-phosphate (PLP), an electrophilic catalyst, for activity. PLP dependent enzymes form covalent Schiff base intermediates with their substrates, which help to labilize functional groups like –COOH

in the case of ODC. The amino acid sequence of this enzyme displays over 90% identity among mammalian species (McCann & Pegg, 1992). This suggests that this enzyme's structure and function are also quite similar among mammalian species. The enzyme's substrate, ornithine, is present in human plasma and is also a product of the urea cycle (Morgan, 1998). Ornithine used for the synthesis of putrescine via ODC may be drawn from these sources.

Mammalian cells and fungi that lack a complete urea cycle may utilize the enzyme arginase to yield putrescine via a two-step process. Arginase first cleaves the guanidine group from L-arginine to form L-ornithine and urea (Fig. 1-2). ODC then converts ornithine to putrescine as described above. In animals, the decarboxylation of ornithine is the only possible synthetic route for putrescine, whereas in bacteria and higher plants, both arginase and ODC pathways are involved in the production of putrescine (Bagni & Tossoni, 2001).

Putrescine can also be synthesized from arginine via enzymes different from arginase. These reactions typically occur in bacteria and higher plants, but not mammalian cells (Bagni & Tossoni, 2001). Initially, ADC removes the –COOH functional group from L-arginine to form agmatine (Fig. 1-2). Then, agmatine is hydrolyzed by agmatinase to form putrescine and urea.

In plants, an additional route to putrescine also exists. After agmatine is derived from arginine as described above, it is converted to *N*-carbamoylputrescine and NH_3 by AIH. *N*-carbamoylputrescine is then hydrolyzed by *N*-carbamoylputrescine amidase to yield NH_3, CO_2, and putrescine (Morgan, 1998). Clearly, the synthesis of putrescine follows a rather elaborate scheme that is not limited to a single route. Multiple routes to

the formation of this polyamine attest to its requirement in normal cell function among a variety of species.

Formation of Spermidine and Spermine

S-adenosylmethionine (AdoMet) normally serves as a substrate involved in methylation reactions in the biosynthesis of amino acids and in capping procedures during eukaryotic transcription (Stryer, 1995). S-adenosylmethionine can also serve as a substrate for S-adenosylmethionine decarboxylase (AdoMetDC). AdoMetDC utilizes a covalently bound pyruvate as its prosthetic group instead of PLP (as in the case of ornithine decarboxylase). This enzyme removes the terminal –COOH group from AdoMet to form decarboxylated S-adenosylmethionine (dcAdoMet) (Fig. 1-3a). Once S-adenosylmethionine is decarboxylated, it is committed to a role in polyamine biosynthesis, unable to participate as a substrate in methylation reactions (Shantz & Pegg, 1999). Instead, dcAdoMet becomes the substrate for the next enzyme in the polyamine biosynthetic pathway, putrescine aminopropyltransferase (PAPT), also called spermidine synthase.

The active form of PAPT is a dimer of two identical subunits. This enzyme has also been purified from various microbial, plant and mammalian sources. Unlike ODC and AdoMetDC, spermidine synthase does not require an enzyme cofactor (Pegg et al., 1995). Spermidine synthase is responsible for transferring the aminopropyl group from dcAdoMet to putrescine (Fig. 1-3b). In this manner, spermidine is formed along with 5'-methylthioadenosine as a side product.

Finally, spermine synthase (SAPT) transfers an aminopropyl group from an additional molecule of dcAdoMet to spermidine resulting in the formation of spermine.

Like spermidine synthase, spermine synthase also consists of two identical subunits of equal size. Though they catalyze nearly identical reactions and are similar in size, PAPT and SAPT are quite specific in that they require putrescine and spermidine as substrates respectively (Pegg et al., 1995).

Polyamine Catabolism

As stated earlier, cells must maintain a careful balance of each polyamine in order to function properly. Each reaction described above for the formation of each polyamine is essentially irreversible. Thus, an additional set of enzymes is required to convert any of the polyamines back to its precursor(s) in order to maintain proper concentrations. Both spermidine and spermine can be acetylated by spermidine/spermine N^1-acetyltransferase (SSAT). This enzyme appears to be ubiquitous in mammalian tissues as well (Casero & Pegg, 1993). The nucleotide sequences for this enzyme from human, hamster, and mouse are more than 95% homologous. This homology as well as conservation among various species may indicate the potential importance of this protein in cellular function (Morgan, 1998). Similarly, this homology may indicate a necessary amino acid sequence required for the structure and functionality of this enzyme. SSAT is the rate-limiting step of the polyamine catabolic pathway. Intracellular activity of SSAT increases in response to elevated levels of polyamines within cells (Casero & Pegg, 1993). Induction of SSAT reduces the concentrations of spermidine and spermine within a cell, thus preventing the cytotoxic levels of polyamines from accumulating (Shantz & Pegg, 1999). An acetyl group from acetyl-coenzyme A is transferred to an aminopropyl group of either spermidine or spermine by SSAT (Fig. 1-3b). The acetylated polyamines are then modified further as described below.

In 1977, Hölttä used the term "polyamine oxidase" to describe various enzymes that complete the back conversion to spermidine and putrescine by oxidizing N^1-acetylspermidine or N^1-acetylspermidine. Typically, oxidases are enzymes that catalyze oxidation reactions in which molecular oxygen is the electron acceptor but oxygen atoms do not appear in the oxidized product. Seiler designates the oxidases as follows: (1) polyamine oxidase (PAO), (2) diamine oxidase (DAO), (3) serum amine oxidases (SAO), (4) monoamine oxidase (MAO) (Seiler, 2000). As the acetylated polyamines are cleaved at secondary amino nitrogens, spermidine or putrescine and 3-acetamidopropronal are released (Fig. 1-3b). Polyamine oxidase is a flavin adenine dinucleotide (FAD) dependent enzyme whereas the diamine oxidases utilize Cu(II) as a cofactor (Thomas & Thomas, 2001). The polyamine oxidases primarily act on the N^1-acetylated polyamines and spermine. The diamine oxidases catabolize diamines such as putrescine, cadaverine and histamine as well as Spd and Spm. Serum amine oxidase also contains copper and catalyzes the deamination of Spd and Spm. The product of spermidine and spermine catabolism, 3-acetamidopropronal, can be further metabolized to both β-alanine and γ-aminobutyric acid (Seiler, 2000).

Polyamines-Functions and Roles

It is quite clear that the path to synthesizing the major polyamines *in vivo* is intricate and detailed. An equally intricate pathway exists, complete with its own set of enzymes, in order to catabolize and maintain the required amounts of these polyamines within a cell. Undoubtedly, the polyamines must be required for some unambiguous, definitive function. However, this exact unambiguous function is yet unknown. In

addition to being regarded as essential for normal cell growth and proliferation, polyamines have been shown to be involved in numerous biological processes.

Spermidine and spermine are tri- and tetracations respectively at physiological pH. With a net positive charge of three and four, these two polyamines are considered the most cationic small molecules within a cell (Davis et al., 1992). As such, these compounds interact with a variety of anionic molecules and cellular structures. These polyamines bind to polyanionic molecules such as DNA, RNA, and phospholipids (Igarashi et al., 1982). Polyamines have a distributed charge due to their carbon backbones. This spacing may allow polyamines to stabilize the conformation of DNA and guard against denaturation (Marton & Morris, 1987). Putrescine, spermidine and spermine can increase the melting temperature of DNA by as much as 40 °C in low salt buffers (Tabor, 1962; Thomas & Bloomfield, 1984). Polyamines appear to also have roles in embryonic development, the cell cycle, cancer, neurochemistry, and pulmonary and immune system functions (Thomas & Thomas, 2001).

Enzyme Inhibition

Researchers have attempted to elucidate the functions of polyamines in a variety of manners, the first of which is through the inhibition of the enzymes involved in the polyamine biosynthetic pathway. Inhibition of the synthetic enzymes leads to decreased concentrations of Put, Spd, and Spm. Inhibition of the enzymes involved in catabolizing the polyamines leads to over accumulation of the polyamines within cells. Developing inhibitors specific for each enzyme in the pathway has proved challenging and has resulted in numerous publications regarding potential polyamine function. Some

inhibitors of the polyamine biosynthetic pathway are discussed below and are reviewed elsewhere (Marton & Pegg, 1995).

The most targeted of the enzymes in the pathway, from an antineoplastic viewpoint, is ornithine decarboxylase. The most widely used inhibitor developed to target ODC is α-difluoromethylornithine (DFMO). Inhibition of ODC by DFMO leads to a major reduction in the amounts of putrescine and spermidine, causing a significant reduction in cell growth once spermidine is depleted (Marton & Pegg, 1995). The effects of DFMO are considered cytostatic rather than cytotoxic and clinical studies of this compound on human tumors have proved disappointing (Schecter et al., 1987). This apparent failure of DFMO for the treatment of human tumors is attributed to the residual amounts of spermine that remain in cells despite treatment with DFMO (Marton & Pegg, 1995). Also, due to the short half-life of ODC, cancer-like cells can overcome an attempt to block its activity very quickly (Bergeron et al., 1995a).

Polyamine Analogues

Targeting the enzymes involved in the synthesis of polyamines has proved problematic. Another approach to targeting the polyamine biosynthetic network in hopes of a therapeutic outcome is to develop polyamine analogues. In theory, synthetically prepared polyamine-like compounds could be incorporated into and distributed throughout a cell. If appearing enough like the endogenous polyamines, the synthetic analogues would mimic the natural polyamines within a cell. A cell's production of the natural polyamines would decrease and perhaps shut down completely, in effect killing a tumor. Numerous compounds have been synthesized and tested for their impact on polyamine pools and their effect on the polyamine regulatory enzymes (Bergeron et al.,

1995a, 1997a). Extensive studies, including molecular modeling, have also been carried out to determine the relationship between biological activities and structural factors of the polyamines and their analogues. For example, varying the length of the carbon backbone in polyamines changes the distance between nitrogen atoms. In turn, this affects charge distribution within the compound altering its interaction with various anionic compounds in the cell. Furthermore, analogues are also prepared by altering the terminal alkylating groups placed on the polyamines. Terminally dialkylated analogues and homologues of spermine, which exhibit antineoplastic activity against a number of tumor cells lines, both murine and human, have been synthesized in Bergeron's laboratories (Bergeron et al., 1994, 1995a, 1997a). The structural requirement of spermidine in supporting cell proliferation has been well documented. Spermidine homologues with a greater than two-carbon extension compared with spermidine exhibited decreased ability to rescue cells from DFMO treatment (Porter & Bergeron, 1983).

Polyamine analogues have also proved to be potent antidiarrheal compounds (Sato et al., 1991). In the course of human immunodeficiency virus (HIV), many patients experience serious diarrhea (AIDS related diarrhea, ARD). Current treatments involve the application of various antimotility agents and even hormonal therapy. Polyamines have been demonstrated to have a profound effect on the gastrointestinal tract (Tansy et al., 1982). By optimizing the length of methylene backbones to obtain ideal separation between charged centers and by incorporating additional functional groups to allow additional routes for metabolism, polyamine analogues with reduced toxicity have been developed for use against ARD (Bergeron et al., 1996, 2001).

Early studies on the effects of polyamines on whole animals models reported changes in blood pressure and slowing of pulse and respiration depending on the dose and route of administration of the polyamines (Wrede, 1924; Tabor & Tabor, 1956). In addition to studying polyamine analogues as antidiarrheals, Bergeron's group has also assessed polyamine analogues for their effectiveness as antiarrhythmics (Bergeron et al., 1998c). The compounds tested included both polyamine antimetabolites and putrescine mimics. The most active analogues observed were dicationic, tetraamines with pyridine ring substitution on the terminal nitrogens—these compounds were able to reverse the progression of an arrhythmic event induced by isoproterenol in a rat model (Bergeron et al., 1998c).

Polyamines may also play an important role in the central nervous system (CNS). Polyamines can cross the blood-brain barrier in limited quantities but the CNS depends on *de novo* synthesis of Spd and Spm as well (Seiler, 1997). The brain contains the highest activity of spermine synthase. Spm is attributed to the functions of neurons whereas Spd plays a role in the conservation and formation of myelin-rich layers. Unusual concentrations of polyamines in the brain are found during the course of several diseases such as Alzheimer's and schizophrenia. Gilad et al. (1997) have observed increased levels of polyamines in the brains of rats exposed to external stressors. Several *N,N'* terminal dialkylated homologues of the tetraamine spermine have been shown to exhibit activity at the *N*-methyl-D-aspartate (NMDA) receptor-channel complex, a ligand-gated ion channel involved in excitatory neurotransmission in the mammalian CNS (Bergeron et al., 1995b). Though limited, information indicating the specific

involvement of polyamines in neuropsychiatric disorders exists and further attention to the role of polyamines in the CNS is warranted.

The roles of polyamines are indeed numerous and diverse. In some cases, their exact function remains ambiguous. In others, their role is quite well understood. Clearly much insight into the functions and roles of polyamines has been gained over the last few decades. The potential for the development of therapeutic outcomes based on the polyamine biosynthetic network is great and clinical trials testing the effectiveness of polyamine analogues are ongoing (Bergeron et al., 2000).

An Unambiguous Role for the Polyamines

Much has been written on the role of polyamines and their interactions with DNA, RNA, and protein biosynthesis. A severe depletion in polyamine levels in mammalian cells leads to reduced cell growth and even cell death. This may be due in part to the involvement of polyamines in the posttranslational modification of eukaryotic translation initiation factor 5A (eIF-5A). More specifically, it has been shown that the polyamine spermidine is the sole aminobutyl donor required to modify a specific lysine residue at a single position (Lys_{50}, human eIF-5A) to the rare amino acid known as hypusine (Fig. 1-4a). Two enzymes affect this modification: deoxyhypusine synthase and deoxyhypusine hydroxylase. Hypusine is an essential component of the eIF-5A protein and the enzymes involved in the biosynthesis of hypusine have become targets for growth inhibition as well. The remainder of this paper will focus on this unique amino acid hypusine, the only known protein on which this modification occurs (eIF-5A), the enzymes that facilitate the modification, and the methods developed in this laboratory to study this biological system more efficiently and effectively.

Hypusine and Eukaryotic Initiation Factor 5A

Hypusine, a basic amino acid, was first isolated from the homogenates of bovine brain tissue via extraction with trichloroacetic acid followed by extensive ion-exchange chromatography (Shiba et al., 1971). From 200 fresh bovine brains weighing 82.0 kg (obtained from a slaughterhouse), approximately 137 mg of hypusine as the dihydrochloride salt were obtained. The brains were first homogenized in 5-10% trichloroacetic acid in a Waring blender. The homogenates were diluted and centrifuged. Supernatants were filtered through celite and then purified through several ion-exchange columns to isolate basic compounds. Eluates were evaporated to dryness and analyzed by high-voltage paper electrophoresis with ninhydrin development. During this process, an unidentified amino acid was observed. Fractions containing this compound were combined and evaporated to dryness *in vacuo*. The dried residue was dissolved in 90% methanol and 6 mL of ether. Cooling this solution overnight yielded 105 mg of crystalline material. The mother liquor yielded an additional crop of 32 mg of hypusine (Shiba et al., 1971).

Shiba's group proceeded to analyze this unidentified compound by nuclear magnetic resonance (NMR), mass spectrometry (MS), chemical degradation experiments, thin layer chromatography (TLC), and amino acid analysis (Shiba et al., 1971). The structure of this compound was then determined and confirmed to be N^6-(4-amino-2-hydroxy-butyl)-2,6-diaminohexanoic acid. It was given the common name of hypusine because it was composed of both **hy**droxy**pu**trescine and ly**sine** moieties (Fig. 1-4a). The stereochemistry of this molecule was determined to be *S* at the C-2 position and *R* at the C-9 position (Park et al., 1982a; Shiba et al., 1982). The formal nomenclature of hypusine was later revised to (2*S*, 9*R*)-2,11-diamino-9-hydroxy-7-azaundecanoic acid.

Other reports refer to hypusine more simply as N^{ε}-(4-amino-2-hydroxybutyl)lysine (Park et al., 1981).

Once isolated, the distribution of this amino acid was studied in various organs of rats and in the brains of rabbits and oxen (Nakajima et al., 1971). Hypusine was determined in the brain, liver, kidney, muscle, spleen, heart, intestine, uterus, lung, blood, and skin of 6-month old Wistar rats. Approximately 1-8 nmol/g of hypusine were detected in the various organs. A similar concentration range for hypusine was observed in the central nervous systems of rabbits and oxen. The renal clearance of hypusine in human urine was also reported to be over 100 mL/min (Nakajima et al., 1971).

Eukaryotic Translation Initiation Factor (eIF) 5A

Protein synthesis requires the coordinated interaction of greater than 100 macromolecules such as tRNA, mRNA, and ribosomes (Stryer, 1995). In general, protein synthesis consists of three stages—initiation, elongation, and termination. Initiation describes the reactions that precede the formation of the peptide bond between the first two amino acids of a protein within a cell. Initiator tRNA binds to the start signal, AUG in eukaryotes, nearest the 5' end of mRNA. Ribosome subunits join together and begin moving along the mRNA in a step-wise manner. Elongation includes the formation of the first peptide bond between the first two amino acids in the protein sequence followed by subsequent peptide bond formations that synthesize the protein in the 5' (N-terminus) to 3' (C-terminus) direction. Termination of protein sequence elongation occurs when a stop signal is recognized along the mRNA. The completed peptide chain is then released from the ribosome.

Initiation is considered the rate-determining step for protein synthesis. Eukaryotic initiation factors (eIFs) regulate the initiation process and assist with the joining of the 60S and 40S ribosome subunits to form an 80S particle along mRNA. Numerous initiation factors are required during the first stage of protein synthesis. When one or more initiation factors are absent or inhibited, initiation of protein synthesis may not occur or will occur to a much lesser extent.

Eukaryotic translation initiation factor (eIF) 5A is an initiation factor that has the unique distinction of undergoing a post-translational modification that is required for its own activity and for cell proliferation. This modification involves the formation of a unique amino acid known as hypusine exclusively at the Lys_{50} residue in human eIF-5A precursor (Fig. 1-4b). The polyamine spermidine is required for this conversion of lysine to hypusine. An enzyme known as deoxyhypusine synthase catalyzes the coupling of the 4-aminobutyl group from spermidine to the ε-amino group of the Lys_{50} residue to form a deoxyhypusine residue. This is followed by hydroxylation of the deoxyhypusine residue by deoxyhypusine hydroxylase to form the fully functional eIF-5A.

Discovery of eIF-5A

Eukaryotic initiation factor 5A is an 18 kDa protein. This initiation factor has been found in both eukaryotic cells and archaea, but not in eubacteria (Chen & Jao, 1999). The amino acid sequence of this protein is highly conserved from yeast to humans. The 12 amino acids closest to the hypusine modification site are highly conserved among various species as well: Ser-Thr-Ser-Lys-Thr-Gly-**Hypusine**-His-Gly-His-Ala-Lys (Park et al., 1997). The human version of this protein has 154 amino acids in its sequence (Fig. 1-5). First isolated from rabbit reticulocyte proteins, eIF-5A was

categorized as initiation factor M2Bα (Kemper et al., 1976). In 1983, this protein was rediscovered and renamed eIF-4D. It was found to be identical to IF-M2Bα (Cooper et al., 1983). The human cDNA for this protein was cloned in 1989 and the protein was again renamed eIF-5A (Smit-McBride et al., 1989).

In 1981, the metabolic labeling of an 18 kDa protein by radioactive spermidine or putrescine was observed (Park et al., 1981; Chen & Liu, 1981). Researchers incubated Chinese hamster ovary (CHO) cells with ^{3}H-spermidine or [*terminal methylenes*-^{3}H]spermidine and found that the radioactivity was incorporated into one cellular protein (Park et al., 1981). It was determined that this labeling occurred as a result of spermidine acting as an aminobutyl donor in the post-translational modification of a particular protein, which converted a single lysine to the unique amino acid hypusine. This protein, called Hy^{+} (indicating the presence of the amino acid hypusine), was also isolated and purified from human lymphocytes and Chinese hamster ovary fibroblasts. It was found that the Hy^{+} protein exhibited electrophoretic properties identical to those of eIF-4D purified from rabbit reticulocytes (Cooper et al., 1983).

The Biosynthesis of Hypusine Requires Two Key Enzymes: DOHS and DOHH

As stated, the post-translational modification of a single lysine residue to hypusine requires the function of two key enzymes: deoxyhypusine synthase (DOHS, E.C. 1.5.1.–) and deoxyhypusine hydroxylase (DOHH, E.C. 1.14.99.29). Park's group first described the biosynthesis of hypusine as a sequential process (Park et al., 1982a). CHO cells were incubated with [4,5-^{3}H]lysine. After acid hydrolysis of these cells, a small portion of the radioactivity of the cellular protein fraction chromatographed to the same position as unlabeled hypusine. This indicated that lysine was a precursor for

hypusine formation. A similar experiment was carried out in which CHO cells were incubated with either tritium labeled lysine or [*terminal methylenes*-^{3}H]spermidine and a metal chelator, α, α-dipyridyl. After digestion of these cells, the label was found to be incorporated into a protein-bound material with chromatographic properties different than those of hypusine. This material was determined to be the unhydroxylated form of hypusine or deoxyhypusine, N^{ε}-(4-aminobutyl)lysine (Park et al., 1982b). Analysis of the deoxyhypusine-containing protein by 2D-electrophoresis provided evidence that this protein was identical to that of the hypusine-containing protein. Park used this experimental evidence to propose that the formation of hypusine occurred in the following manner: peptide-bound lysine + spermidine → peptide-bound deoxyhypusine → peptide-bound hypusine. Park's group also postulated that a metal-requiring enzyme catalyzed the hydroxylation of deoxyhypusine since hypusine formation was inhibited by the presence of a chelating agent and the effects of this chelating agent were reversed upon addition of ferrous sulfate (Park et al., 1982b).

Deoxyhypusine Synthase

Soon after the above scheme for hypusine formation on a single protein was proposed, the enzymes responsible for this post-translational modification were studied. Deoxyhypusine synthase (DOHS) is the enzyme responsible for the first step in hypusine biosynthesis. Approximately 40-43 kDa in size depending on species, DOHS is an NAD^{+}/NADH dependent enzyme (Chen & Dou, 1988). This enzyme has been purified to homogeneity by substrate elution affinity chromatography from *Neurospora crassa* (Chen & Tao, 1995). DOHS has also been purified and characterized from rat testis and HeLa cells by ammonium sulfate fractionation and ion exchange chromatography (Wolff

et al., 1990; Klier et al., 1995). The enzyme is a homotetramer and its amino acid sequence appears to be highly conserved, especially towards the C-terminal region (Chen & Jao, 1999).

The reaction by which deoxyhypusine synthase converts lysine on precursor eIF-5A to deoxyhypusine can be expressed in four steps (Fig. 1-6). First, the enzyme and its NAD^+ cofactor catalyze the dehydrogenation and oxidative cleavage of spermidine (Wolff et al., 1990). This is followed by the formation of an enzyme-imine intermediate and 1,3-diaminopropane as a side product. The iminobutyl group is then transferred from the enzyme-imine intermediate to the lysine residue on eIF-5A. Lastly, the imino group is reduced to form the deoxyhypusine residue on eIF-5A. As Lys_{50} in the human eIF-5A protein has been identified as the sole residue to undergo this modification to date, Lys_{329} in the human deoxyhypusine synthase enzyme has been identified as the key residue involved in the enzyme-substrate intermediate discussed above (Joe et al., 1997). When Lys_{329} of the enzyme was substituted with alanine or arginine, the enzyme-substrate intermediate formation and DOHS activity was completely eliminated. Mutations at nine other conserved lysine residues within the enzyme (Lys 141, 156, 205, 212, 226, 251 and 338) did not affect enzyme activity in the same manner as mutations at Lys_{329}. It was determined that Lys_{329} is located at the active center of the enzyme near the NAD-binding site and in the middle of the predicted spermidine-binding pocket thus enabling it to accept the aminobutyl group (Joe et al., 1997). Recently, the crystal structure of the human form of deoxyhypusine synthase with bound NAD cofactor has been reported (Liao et al., 1998). The crystal structure observed in this study shows that the entrance to the active site is blocked by a two-turn α-helix. This suggests that the reaction steps

described above are accompanied by significant conformational changes in the enzyme (Liao et al., 1998). Undoubtedly, the crystal form of the enzyme is likely to be in quite a different conformation than it would normally be in solution or *in vivo*. A significant conformation change may occur immediately upon solvation of this enzyme prior to exposure to immature eIF-5A or NAD^+. The establishment of a basis of knowledge on the eIF-5A protein in its solution phase is warranted and therefore pursued in this study.

Deoxyhypusine Hydroxylase

Deoxyhypusine hydroxylase (DOHH) is the second enzyme involved in the biosynthesis of hypusine on eIF-5A. It is responsible for adding a hydroxyl functional group in the *R* configuration to the C-9 position of the deoxyhypusine residue on the eIF-5A intermediate. To date, this enzyme has not been purified to homogeneity. In fact, the study of this particular enzyme has proved to be much more problematic than that of DOHS. The main difficulty in the development of a purification method for this enzyme is the fact that a convenient assay to monitor the hydroxylation reaction is lacking (Chen & Jao, 1999). DOHH has only been partially purified from the homogenates of rat testis (Abbruzzese et al., 1986). Adding to the problem is the fact that the partially purified DOHH does not act on the free amino acid, deoxyhypusine. So, there is currently no synthetic substrate other than deoxyhypusine-containing eIF-5A for this enzyme to work on.

Abbruzzese et al. have proposed several indirect methods for the monitoring of DOHH activity. One method relies on the measurement of tritiated water formed as the deoxyhypusine hydroxylase enzyme displaces a tritium at the C-9 position of deoxyhypusine. Another method requires a dual-labeled protein substrate—both ^{14}C and

^{3}H are incorporated into the side chain of deoxyhypusine by incubating CHO cells with both 1,4-^{14}C putrescine and 2,3-^{3}H putrescine. This is a rather creative method. Theoretically, as DOHH acts upon the dual-labeled substrate, one fourth of the tritium is released whereas all of the ^{14}C is retained. The degree of hydroxylation can be estimated from the change in the ratio of ^{3}H to ^{14}C in the protein fraction (Abbruzzese et al., 1988). Still, these methods do not lend themselves to convenience and reliability. A dually labeled compound may also create cross contamination problems and ^{3}H:^{14}C ratios may not be accurate.

More recently, an assay for deoxyhypusine hydroxylase was developed using HeLa cells incubated with either ^{3}H-spermidine or ^{14}C-spermidine and a metal chelator (Csonga et al., 1996). Metal chelators prevented DOHH from completing the hydroxylation of deoxyhypusine on eIF-5A and in the process provided deoxyhypusine-containing eIF-5A, the natural substrate to form hypusine-containing eIF-5A. In the process, the dependency of this enzyme on a metal ion, Fe^{3+}, was determined. Hypusine-containing eIF-5A can be oxidized by periodate to yield lysine-containing-eIF-5A (eIF-5A precursor), formaldehyde and β-aminopropionaldehyde. These aldehydes can then be extracted and measured for their radioactivity. Oxidation will theoretically only occur in the presence of the hypusine containing eIF-5A (-OH at C-9) and therefore the radiolabeled byproducts of this oxidation will be proportional to the activity of DOHH. Others are attempting to refine this technique in order to fully purify deoxyhypusine hydroxylase (Chen & Jao, 1999). At the time of this writing, no further progress has been reported regarding the full purification and characterization of deoxyhypusine hydroxylase.

Hypusine and eIF-5A: Functions and Roles

How important is hypusine? What role does eIF-5A play? Clearly these are important questions. It should be obvious that this unique amino acid, protein, and the enzymes that synthesize them play a key role in nature. Two enzymes have been devoted to the process of producing this unique amino acid on a single residue of a single protein. The highly conserved nature of the amino acid sequence in the protein also suggests a vital cellular role. Like the polyamines, the exact role for free and protein-bound hypusine and its parent protein are as of yet unknown. Also like the polyamines, numerous studies have been conducted to elucidate the functions of these biological molecules. The main pathway taken to determine the functions of hypusine and eIF-5A has been that of developing inhibitors of the two key enzymes involved in the formation of hypusine—DOHS and DOHH. In this manner, much insight has been gained into the functions and roles of hypusine and eIF-5A. In the same manner, these same studies have made solving the puzzle of hypusine and eIF-5A more difficult as well.

Hypusine and the hypusine-containing eIF-5A protein are essential in cell proliferation (Park et al., 1993, 1997). Early on, a correlation was observed between hypusine synthesis and cell growth. Hypusine was shown to be essential for the *in vitro* activity of eIF-5A in the stimulation of methionyl-puromycin synthesis (Kemper et al., 1976). This *in vitro* study mimics the formation of the first peptide bond in protein synthesis. However, another study showed that protein synthesis in yeast continued even after introducing a gene variant that depleted eIF-5A (Kang et al., 1993). Studies have shown that preventing the synthesis of mature, hypusine-containing eIF-5A by blocking the synthesis of its aminobutyl donor spermidine also leads to the arrest of growth in eukaryotic cells (Park et al., 1997). Hypusine synthesis is suppressed in cells where

spermidine has been depleted and, upon replenishment of spermidine or a suitable spermidine analogue, hypusine synthesis is dependent upon the amount of spermidine or analogue that has been supplemented (Marton & Pegg, 1995; Tome & Gerner, 1997).

Despite the fact that the exact cellular function(s) of eIF-5A are still unknown, it is clear that cellular growth is adversely affected in its absence and in the absence of its hypusine residue. Studies involving *S. cerevisiae* show that total protein synthesis is only reduced to 30% of normal levels in eIF-5A deficient cells (Kang & Hershey, 1994). This evidence suggests that eIF-5A and hypusine are not required solely for protein synthesis. In any case, there is an undeniable link between polyamines, eIF-5A, and hypusine and hypusine is absolutely required for eIF-5A to be completely functional, whatever function that may be.

Several inhibitors of the enzymes, as well as analogues of the substrates, involved in the biosynthesis of hypusine on eIF-5A have been employed in order to gather more information on the function of these molecules. A number of spermidine analogues have been synthesized as described above in hopes of discovering an effective antineoplastic drug. Several inhibitors of the polyamine biosynthetic pathway have also been proposed (e.g. DFMO). It is possible that these analogues and inhibitors function as antiproliferatives due to the fact that by depleting the natural source of spermidine, the aminobutyl donor required for hypusine synthesis on eIF-5A is also depleted.

The most potent spermidine analogue reported thus far is N^1-guanyl-1,7-diaminoheptane (GC_7), which causes growth arrest of mammalian and archaea cells (Jakus et al., 1993; Jansson et al., 2000). It is thought that this compound competes with spermidine for DOHS. Metal chelators have also been used as inhibitors of

deoxyhypusine hydroxylase. Compounds like mimosine (3-(*N*-(3-hydroxypyridin-4-one))-2[S]-aminopropionic acid) reportedly cause an arrest in cell proliferation at the G1/S boundary of the cell cycle (Hanauske-Abel et al., 1995). Blocking S-phase entry interferes with the onset of DNA replication and therefore hints that eIF-5A may play a role in this manner. Studies such as these are complicated by the fact that chelators such as mimosine have exhibited other cellular functions such as interfering with DNA synthesis by inhibiting deoxyribonucleotide metabolism (Gilbert et al., 1995). Methylglyoxal bis(guanylhydrazone) (MGBG) is also a reported inhibitor of AdoMetDc (Marton & Pegg, 1995). This inhibitor prevents the decarboxylation of S-adenosylmethionine therefore cutting off the supply of dcAdoMet required for the synthesis of spermidine. Elimination of spermidine can prevent the formation of hypusine by removing the aminobutyl donor source.

It should be noted that the inhibitors used to study the hypusine modification on eIF-5A lack specificity, like mimosine, and exhibit general toxicity as well, like DFMO, MGBG, and GC_7. Caution should be taken when interpreting the results obtained to date, as when examined individually they may not tell the complete story of eIF-5A and hypusine. Undoubtedly, better tools are required to study this biological system. The crystal structure of DOHS obtained by Liao's group in 1998 should provide a template for the development of more specific inhibitors of this enzyme. Tools developed in Bergeron's laboratory, to be discussed later in this paper, should also aid in the elucidation of the functions and roles of hypusine and eIF-5A.

The decrease or shutdown of cellular proliferation as a result of the prevention of eIF-5A/hypusine formation has encouraged further study of this biological system as a

target for antineoplastic development. Other studies have linked hypusine and eIF-5A function to human immunodeficiency virus type 1 (HIV-1). EIF-5A has been shown to interact with Rev, a viral regulatory protein essential for HIV-1 replication (Ruhl et al., 1993). Rev functions by activating the nuclear export of unspliced (intron-containing) viral mRNAs (Pollard & Malim, 1998). Rev is a 13 kDa RNA binding protein. It contains at least three functional domains: an arginine-rich domain, a nuclear export signal (NES), and a homomultimerization domain (Hope, 1999). The arginine-rich domain of the Rev protein allows it to bind specifically to a 240-base region of complex RNA secondary structure called the Rev response element (RRE). RNA gel mobility shift assays have indicated the interaction between RRE RNA and both deoxyhypusine- and hypusine-containing eIF-5A protein (Liu et al., 1997). No interaction is observed with the unmodified, lysine-containing eIF-5A protein. From this observation, it is clear that eIF-5A may function as a cellular cofactor involved in the recognition and nuclear export of certain mRNA in addition to its potential role in protein synthesis (Park et al., 1997). From this observation, a potential antiviral therapeutic window also arises. If the interaction of mature eIF-5A, Rev, and RRE can be prevented, it is conceivable that HIV replication can be halted. Indeed, mutant forms of eIF-5A that form a complex with Rev and RRE were reported to inhibit HIV-1 replication in human lymphocyte cell lines (Bevec et al., 1996). Several studies have characterized the interactions between Rev and RRE by NMR (Peterson, et al., 1994, 1996; Battiste, et al., 1995, 1996). To date, no studies have examined the interactions between Rev and RRE with eIF-5A by NMR. Future study regarding eIF-5A and hypusine function is warranted as it offers a potential antiviral therapeutic outcome.

Isolation and Identification of Hypusine Dipeptides

GABA or γ-aminobutyric acid is an abundant constituent of the brain. It is the major inhibitory neurotransmitter in the mammalian central nervous system (Siegal et al., 1994). GABA meets the five classical criteria for assignment as a neurotransmitter: (1) it is present in the nerve terminal, (2) it is released from electrically stimulated neurons, (3) there is a mechanism for reuptake of the released neurotransmitter, (4) its application to target neurons mimics the action of inhibitory nerve stimulation, and (5) specific receptors for GABA exist (Siegal et al., 1994). The effects of this neurotransmitter are widespread. A variety of evidence suggests a role for altered GABA function in several neurological and psychiatric disorders of humans such as epilepsy, schizophrenia, sleep disorders, and Parkinson's disease. Also, recall that GABA and β-alanine are terminal catabolites of polyamine metabolism as well (Morgan et al., 1998).

α-(γ-aminobutyryl)-hypusine

In light of the fact that both GABA and hypusine are abundant in the brain, there should be ample opportunity (and enzymes no doubt) to couple the two compounds together to form a dipeptide. A novel GABA-containing peptide was isolated from bovine brain and determined to be α-(γ-aminobutyryl)-hypusine, or GABA-hypusine (Fig. 1-7) (Sano et al., 1986). Bovine brains (1.2 kg) obtained from slaughterhouses were dissected, boiled in water and then homogenized and extracted with trichloroacetic acid. Extracts were centrifuged and supernatants were filtered through a series of ion exchange columns in order to isolate basic aliphatic amino acids. High-voltage paper electrophoresis, TLC, and HPLC techniques were used to examine column eluates. Recall that the amino acid hypusine was isolated and identified in a similar manner by

this same research group. Fractions collected after hypusine was detected yielded an unknown compound. Two ninhydrin positive substances were observed when the acid hydrolysate of this unknown was examined by electrophoresis and TLC. The TLC R_f values in three different solvent systems were the same as those for hypusine and GABA. In a similar manner, migration distances in high-voltage electrophoresis matched those for hypusine and GABA as well. Analysis of the acid hydrolysate of the unknown compound by HPLC indicated approximately equimolar amounts of GABA and hypusine. It was determined by HPLC that approximately 0.9 μmol of α-(γ-aminobutyryl)-hypusine was isolated from 1.2 kg (0.75 nmol/g) of bovine brain. The structure of the isolated dipeptide was verified by comparison with a synthetic version of GABA-hypusine. Accounting for loses due to purification, the authors estimated the actual concentration of GABA-hypusine in bovine brain to be > 1 nmol/g.

Further study by this same group determined that GABA-hypusine has a distribution restricted to the brain (Sano et al., 1987). The peptide was detected in the brain tissues of the rat, rabbit, ox, and monkey. The dipeptide was not detected in the brain of the dog or in a specimen of cat brain. Concentration of the dipeptide in the various brain tissues ranged from 1.3 to 9.5 nmol/g wet weight (Sano et al., 1987).

α-(β-alanyl)-hypusine

β-alanine has also been isolated in the form of a hypusine dipeptide: α-(β-alanyl)-hypusine (Fig. 1-7) (Ueno et al., 1991). The same group responsible for the isolation of hypusine and GABA-hypusine accomplished this work using a procedure similar to that described above. Approximately 1 μmol of this dipeptide was isolated from 1090g of bovine brain. Two ninhydrin positive substances were also produced upon

acid hydrolysis of this compound. The two substances were determined to be β-alanine and hypusine by TLC, HPLC, and high-voltage electrophoresis analysis.

Several dipeptides containing an ω-amino acid and a basic amino acid have been identified and divided into two groups: those that contain GABA and a basic amino acid and those that contain β-alanine and a basic amino acid. In addition to GABA-hypusine discussed above, the following GABA containing dipeptides have been found in mammalian brain: homocarnosine (or GABA-histidine), homoanserine (or GABA-1-methylhistidine), GABA-lysine and GABA-cystathionine. In addition to α-(β-alanyl)-hypusine discussed above, the following β-alanine containing dipeptides have been found in mammalian brain: carnosine (α-(β-alanyl)histidine), anserine (α-(β-alanyl)-1-methylhistidine), and α-(β-alanyl)lysine.

The function of these dipeptides is not known. All that is known is that these compounds are found in excitatory tissues such as nervous system and muscle. Carnosine is rich in olfactory neurons and is considered to have a neurotransmitter role in the olfactory system (Sano et al., 1986). An enzyme known as carnosine synthetase was shown to catalyze the synthesis of dipeptides containing both ω-amino acids and basic amino acids (Sano et al., 1987).

The isolation and identification of the two hypusine dipeptides discussed above has currently only been accomplished by a single group of researchers. Their last published report regarding these compounds was published over 10 years ago (Ueno et al., 1991). There is the distinct possibility that many more of these hypusine-containing peptides have yet to be isolated. There also remains the possibility that these hypusine dipeptides discovered were actually artifacts of the extraction process used. In other

words, it is possible that GABA- and β-ALA-hypusine are dipeptide sequences from yet another hypusine-containing protein. Clearly, more work in this area is merited and better synthetic and analytical tools are required to accomplish this task.

Proposal

In order to understand and determine the functions of hypusine, eIF-5A, and the novel dipeptides, α-(γ-aminobutyryl)-hypusine and α-(β-alanyl)-hypusine, it is imperative that reliable and efficient techniques be developed to synthesize and analyze these compounds. The proper tools to carry out these experiments are lacking. Hypusine, deoxyhypusine, hypusine dipeptides, and eIF-5A peptide fragments cannot be purchased from commercial sources. Additional syntheses and analyses of these compounds are required. In this study, the synthesis and analysis of hypusine dipeptides, ^{3}H-hypusine, and the incorporation of hypusine, deoxyhypusine, and ^{13}C labeled hypusine reagents into eIF-5A peptide fragments has been explored. A new tissue extraction method for the analysis of free hypusine and hypusine dipeptides from rat brain has also been developed. Finally the complete characterization of eIF-5A peptide fragments containing lysine, deoxyhypusine, hypusine, and ^{13}C labeled hypusine (position 50 in human eIF-5A) by both 1D and 2D ^{1}H and ^{13}C NMR experiments were completed in order to establish a basis of NMR data for the 20 amino acid region of eIF-5A immediately surrounding the lysine to hypusine modification site.

A synthetic scheme reported by Bergeron for the preparation of a hypusine reagent has been used as the basis for the synthesis of the hypusine-containing dipeptides described in this study (Bergeron et al., 1997b, 1998a). This synthetic scheme is quite versatile due to the presence of orthogonal protecting groups. Thus, the fully protected

dipeptide was achieved via the selective removal of a BOC protecting group from the hypusine reagent (Gibson et al., 1994) followed by the coupling of BOC-GABA or BOC-β-alanine to the free N^{α} amine. Protecting groups were removed with TFA and TIS and the compounds were purified via ion exchange column. The compounds α-(γ-aminobutyryl)-hypusine and α-(β-alanyl)-hypusine have been synthesized and characterized by TLC, MS, optical rotation and NMR. To date, only a brief mention of a synthetic scheme to access GABA-hypusine in low yields (5%) has been reported (Sano et al., 1986, 1987). In addition, no synthetic scheme for the synthesis of α-(β-alanyl)-hypusine has been described prior to this. The synthetic scheme described in Chapter 2 allows access to the GABA- and α-(β-alanyl)-hypusine dipeptides from Bergeron's hypusine reagent with a 46-48% overall yield. In addition to increased yields of these compounds, the natural stereochemistry of the hypusine moiety (2*S*, 9*R*) is maintained throughout the synthesis as a result of steps to prevent racemization from occurring (Bergeron et al., 1998a).

Another hindrance to the study of hypusine and hypusine containing dipeptides is the lack of a routine pre-column derivatization HPLC assay to detect these compounds. Synthetic routes to these compounds provide standards to use for the development of such an assay. The use of OPA/NAC as a precolumn derivatization agent for the routine separation of hypusine, α-(β-alanyl)-hypusine, and γ-(aminobutyryl)-hypusine has been explored and compounds can be routinely detected below 0.5 nmol (λ_{ex} = 340 nm, λ_{em} = 440 nm). The successful development of an HPLC analytical technique to detect these standards can then be adapted for the routine analysis of these compounds in animal

tissues, such as rat brain. The precolumn derivatization of hypusine and its dipeptides has also been successfully adapted to an HPLC-MS system (Chapter 5).

To be able to routinely assay for hypusine and its dipeptides in the free form (non-protein bound) in animal tissues such as rat brain, an efficient tissue extraction method must also be developed. It is important to subject the tissues to conditions that will only extract the free form of the compounds and not hydrolyze them from their parent proteins (i.e. hypusine from eIF-5A). Key to this method is the availability of a tracer compound that can be used to develop and gauge the efficiency of the extraction method. Again the versatility of the hypusine reagent synthetic scheme described in Chapter 2 lends itself to the incorporation of a tritium label into the hypusine molecule. The $^3H_2$ gas reduction of a terminal nitrile will incorporate a tritium label into the hypusine molecule at the C-11 position. The use of this novel ^{3}H-hypusine as a tracer for the development of an efficient tissue extraction method for free hypusine and hypusine dipeptides has been explored (Chapter 5). Tissues were homogenized and extracted with 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 10.7) followed by centrifugation and neutralization of the supernatant. The extract was then lyophilized followed by purification through G-25 sephadex before HPLC analysis. Combining the extraction method developed using the ^{3}H-hypusine tracer compound with the OPA/NAC HPLC assay has allowed for the detection of compounds in rat brain tissue that coelute with both hypusine and GABA-hypusine standards. HPLC-MS analysis was able to elucidate the identity of one of the suspect peaks as hypusine.

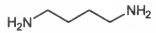
In addition, the same synthetic scheme used to access the dipeptides and ^{3}H-hypusine is also versatile enough to allow the incorporation of a ^{13}C label into the

hypusine reagent at the C-11 position (Chapters 2 & 4). These hypusine reagents were then used in solid phase peptide synthesis (SPPS). Prior to this dissertation, hypusine reagents have been successfully incorporated into hexapeptides (Bergeron et al., 1997b, 1998a). Using FMOC solid phase peptide chemistry synthetic techniques, hypusine and deoxyhypusine reagents, as well as a ^{13}C labeled hypusine reagent were successfully incorporated into 20 and 21 amino acid peptide sequences matching the hypusine-containing region of the human eIF-5A protein, Cys_{38}-Leu_{58} (Chapter 3). Furthermore, these peptides have been characterized by HPLC, MALDITOF-MS, and amino acid sequencing. Each peptide synthesized has a purity ranging from 85-99% and no deletion sequences were detected.

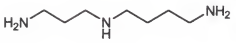
Both 1D and 2D NMR studies (^{1}H and ^{13}C) on eIF-5A peptide fragments have been completed as well (Chapter 6). The integrity of the ^{13}C labeled reagent was assessed via ^{13}C NMR experiments. The presence of a ^{13}C labeled carbon within a large peptide was easily identified in the NMR spectrum. Other NMR techniques such as TOCSY (Total Correlation Spectroscopy), NOESY (Nuclear Overhauser Effect Spectroscopy), and HMQC (Heteronuclear Multiple Quantum Correlation spectroscopy) were used to help assign the NMR spectrum of each eIF-5A peptide fragment as fully as possible. The synthesis and analysis of these eIF-5A peptide fragments may provide a more detailed understanding of the amino acid region surrounding the hypusine modification site in the eIF-5A protein. Prior to this dissertation, no NMR data for hypusine-containing eIF-5A peptides up to 20 amino acids in length has been acquired.

The hypusine reagent scheme described by Bergeron is indeed a powerful synthetic tool. This study has capitalized on that versatility in order to access previously

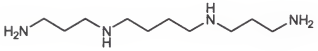
unavailable synthetic compounds and develop new analytical techniques for their analysis. The ability to synthesize, analyze, and detect hypusine containing compounds will indeed provide additional, necessary tools that will aid the complete understanding of the functions and roles of the unique amino acid hypusine, its dipeptides, and the single protein on which hypusine is formed via a post-translational modification, eIF-5A.



putrescine
1,4-butanediamine
"4"



spermidine
N^1-(3-aminopropyl)-1,4-butanediamine
"3-4"



spermine
N^1, N^4-bis(3-aminopropyl)-1,4-butanediamine
"3-4-3"

1,3-propanediamine
"3"

cadaverine
1,5-pentanediamine
"5"

Figure 1-1. The Polyamines

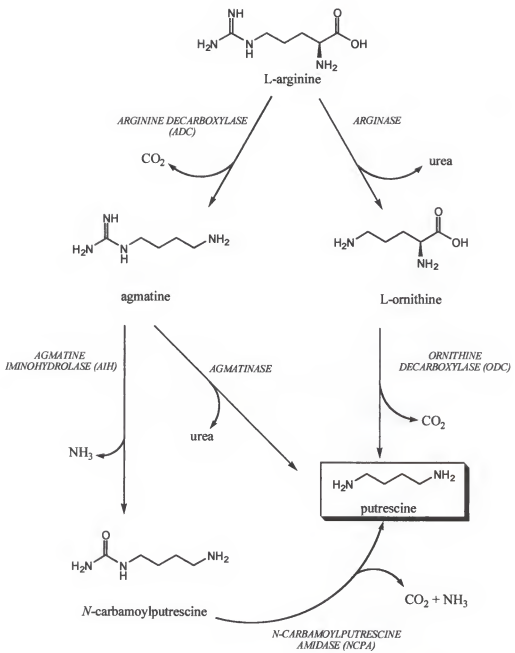


Figure 1-2. Polyamine Biosynthetic Pathway I–Access to Putrescine

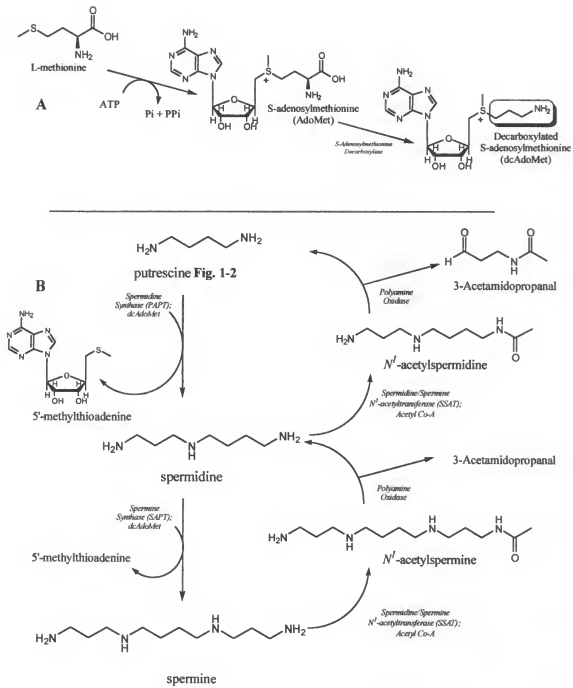


Figure 1-3. Polyamine Biosynthetic Pathway II–A) dcAdoMet and B) Spermidine and Spermine

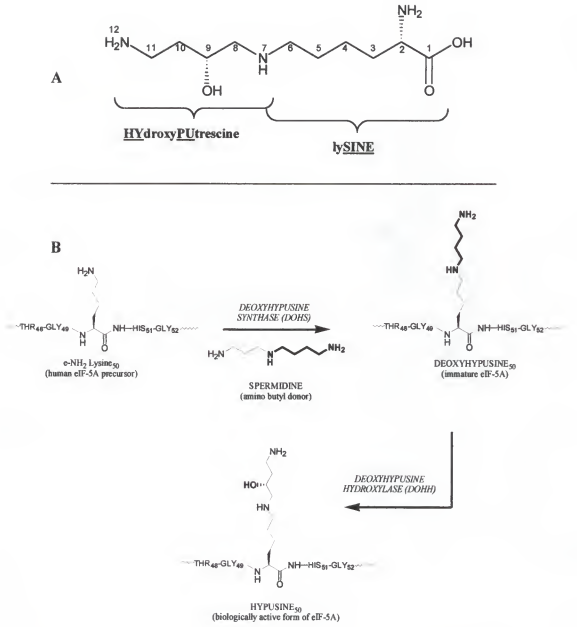
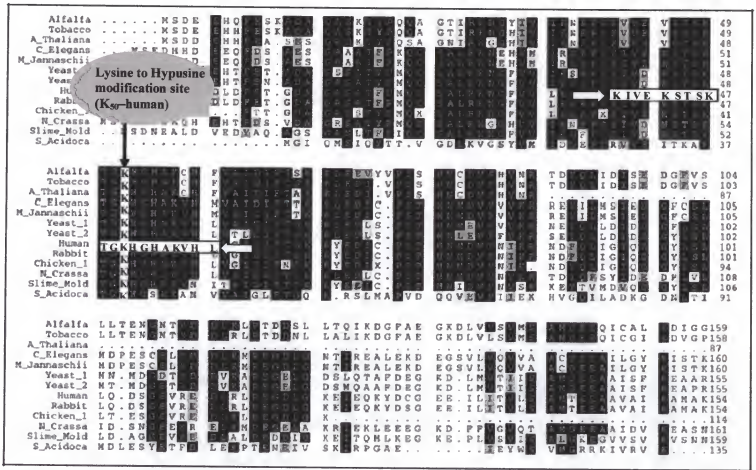
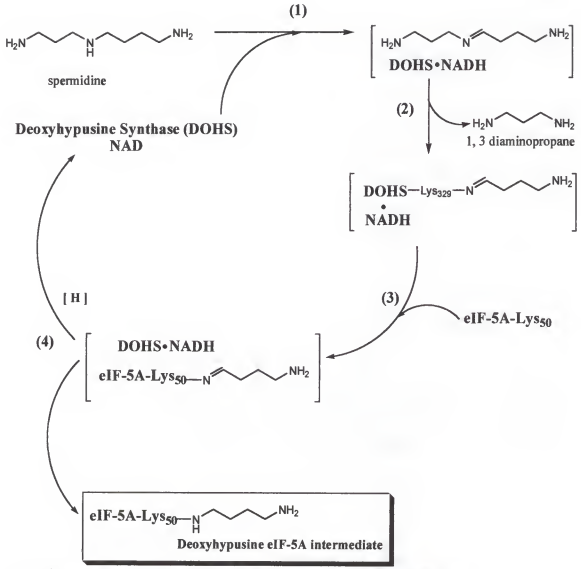


Figure 1-4. Post-Translational Modification of Lysine to Hypusine–A) Hypusine and B) Hypusine Biosynthesis on eIF-5A



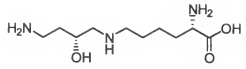
(Shaded blocks indicate conserved regions. Arrows indicate region of human eIF-5A surrounding Lys50 Hypusine Modification Site-Chen et al., 1999)

Figure 1-5. Eukaryotic Initiation Factor 5A Amino Acid Sequence (No Hypusine Modification)



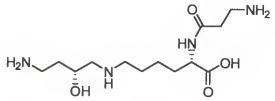
1 - NAD^+ cofactor catalyzes the dehydrogenation and oxidative cleavage of spermidine
2 - Formation of an enzyme-imine intermediate and 1,3-diaminopropane side product
3 - Transfer of iminobutyl group from the intermediate to the lysine residue on eIF-5A
4 - Reduction to form the deoxyhypusine residue on eIF-5A
(Joe et al., 1997)

Figure 1-6. Deoxyhypusine Synthase (DOHS)–Modification of Human eIF-5A (Lys_{50})



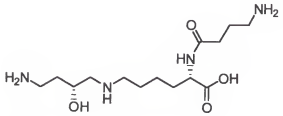
$C_{10}H_{23}N_3O_3$
Mol. Wt.: 233.31

Hypusine



$C_{13}H_{28}N_4O_4$
Mol. Wt.: 304.39

α–(β-alanyl)-hypusine



$C_{14}H_{30}N_4O_4$
Mol. Wt.: 318.41

α–(γ-aminobutyryl)-hypusine

Figure 1-7. Hypusine and Hypusine-Dipeptides

CHAPTER 2
SYNTHESIS I–SOLUTION PHASE

Previous Synthetic Methods for Hypusine

The importance of obtaining a synthetic route to hypusine in order to study this unique amino acid more carefully has been established in the previous chapter. One initial reported synthesis of this amino acid was based on the coupling of L-lysine and 4-amino-1-bromo-2-butanol with the secondary alcohol component being derived from malic acid (Shiba et al., 1982). Both isomers of hypusine, (2*S*, 9*R*) and (2*S*, 9*S*), were prepared as crystalline dihydrochloride salts in this manner. It was determined that (2*S*, 9*R*)-2,11-diamino-9-hydroxy-7-azaundecanoic acid, or (2*S*, 9*R*) hypusine, was identical with naturally occurring hypusine in all respects (Shiba et al., 1982).

In response to the growing interest in hypusine and its origin, Tice and Ganem (1983a) reported the preparation of functionalized hydroxyputrescine-aldehyde adducts to use in the synthesis of hypusine. Furthermore, Tice and Ganem (1983b) also reported an additional synthesis of hypusine after retrosynthetic analysis of the compound suggested that the coupling of the N7-C8 bond might be prepared via reductive amination of a cyclic aldehyde with a protected form of lysine. Also, the use of dibenzyltriazones as amino protecting groups and a D-asparagine amino derivative were demonstrated to be useful in the syntheses of hypusine, deoxyhypusine, spermidine, and spermidine analogues (Knapp et al., 1992).

Hypusine was obtained in low yields by Shiba's pathway (Shiba et al., 1982). Ganem's method required the cumbersome separation of diastereomers (Tice & Ganem,

1983b). These hindrances prompted Bergeron's group to explore alternative methodologies that would allow easier access to hypusine and related compounds. An initial strategy focused on the design of an electrophilic 4-amino-2-hydroxybutane synthetic reagent that would allow the necessary four carbon extension on the lysine residue while simultaneously providing a hydroxyl group and a terminal amino group (i.e. hydroxyputrescine moiety) (Bergeron et al., 1993). Commercially available (*R*) and (*S*) epichlorohydrins were used as chiral epicyanohydrin precursors in this study. Reaction of the epichlorohydrins with an *Nε*-benzyl-*Nα*-carbobenzoxy-L-lysine benzyl ester yielded the (2*S*, 9*S*) chlorohydrin and oxirane in 75% and 8% yield respectively. The chloride ion of the chlorohydrin compound was then displaced by cyanide. This was followed by the simultaneous reduction of the nitrile and removal of the benzyl- and carbobenzyloxy-protecting groups via hydrogenation with PtO_2 (catalyst) in acetic acid. Neutralization gave the hypusine freebase and reacidification yielded (2*S*, 9*R*)-(+)-hypusine dihydrochloride for an overall 53% yield (Bergeron et al., 1993).

Preparation of Hypusine Reagent

In order to study hypusine in a peptide environment, particularly within an eIF-5A peptide sequence surrounding the Lys_{50}-Hyp_{50} (human eIF-5A) modification site, a useful "hypusine reagent" is required. This reagent would also have orthogonal protecting groups, which would allow the amino acid to be coupled to adjacent residues in a peptide sequence and at the same time protect the hypusine side chain from undergoing modification during the coupling process. These protecting groups should also be readily removed under conditions that will result in the maximum yield of the unprotected peptide while avoiding undesirable cleavage of the peptide bonds. Additionally, the ideal

hypusine reagent would be suitable for both traditional solution phase peptide chemistry as well as more recent solid phase peptide synthetic methodologies, including both manual and automated techniques.

Successful preparation of hypusine with relatively good yields led to the development of both hypusine and deoxyhypusine reagents that could be incorporated into peptide syntheses (Bergeron et al., 1997a, 1998a). The synthesis described below (Bergeron et al., 1998a) and displayed in Figure 2-1 is the basis for the preparation of additional hypusine and deoxyhypusine containing peptides to be discussed in Chapter 3. More detailed synthetic data (i.e. reagent amounts, yields, temperature conditions, etc.) for the precursors prepared in this study via the schemes presented by Bergeron are given in Chapter 4.

The first step in the synthesis of the hypusine reagent was the protection of the *Nα* of commercially available *Nε*-CBZ-L-lysine tert-butyl ester (BACHEM Bioscience Inc.) with di-*tert*-butyl dicarbonate in aqueous $NaHCO_3$ and chloroform (Fig. 2-1). The fully orthogonally protected lysine reagent (**1**) was prepared from this biphasic reaction. The BOC, CBz, and O-*t*-butyl protecting groups now present on this molecule can be selectively removed under distinctive conditions without affecting the integrity of the other protecting groups. The *Nε*-CBZ group was then removed via hydrogenation with Pd-C catalyst (**2**) followed by the coupling of the now free *Nε* of the lysine compound with (*S*)-(+)-epichlorohydrin in cyclohexane. The precipitated product was filtered and purified to obtain **3**. Benzyl chloroformate was then condensed with the chlorohydrin (**3**) to yield the N^7-CBZ protected compound (**4**). The carbamate linkage at this point in the synthesis prevents azetidinium formation (which can cause racemization at C-9) that was

observed during the initial scale-up of the hypusine reagent synthesis (Bergeron et al., 1998a). Potassium cyanide in the presence of 18-crown-6 was used to incorporate an additional carbon and nitrogen into the compound (**5**). The 18-crown-6 reagent forms a complex with KCN allowing the cyano group to act as a nucleophile and displace the chlorine atom on the chlorohydrin compound (Cook et al., 1974). Though not shown in the scheme presented, compound **5** can be converted to a Mosher ester in order to verify chiral integrity at the C-9 position (Dale & Mosher, 1973). Resonances for the 9*R* and 9*S* methoxy groups resulting from the Mosher reaction are easily observed and distinguishable via ^{1}H NMR (Bergeron et al., 1998a).

The nitrile (**5**, non-Mosher ester) was then reduced with H_2 (g) and a mixed catalyst of Pd-C and PtO_2 to give **6**. Since hydrogenation also removes CBZ protection on N-7 and creates a free terminal amine on N-12, both amines were reprotected with *N*-(benzyloxycarbonyloxy)succinimide (CBZ-ONSu) to yield **7**. The addition of trifluoroacetic acid (TFA) and triethylsilane (as a scavenger) removed both the BOC and *t*-butyl ester protecting groups leaving the di-CBZ amino acid, **8**. A tetrahydropyran (THP) ester was formed at the C-9 position via addition of 3,4-dihydro-2*H*-pyran to the secondary alcohol (**9**). This was done in order to prevent the nucleophilic reactivity of the hydroxyl group during the coupling of the hypusine reagent during solution or solid phase peptide synthesis. Finally, the $N\alpha$ of the reagent was protected with a 9-fluorenylmethoxycarbonyl (FMOC) group by reacting **9** with a solution of 9-fluorenylmethyl *N*-succinimidyl carbonate in DMF and 9% Na_2CO_3 (**10**). This fully protected hypusine reagent (**10**) was then utilized in the solid phase peptide synthesis of hypusine-containing eIF-5A peptide fragments discussed in Chapter 3.

Synthesis of Hypusine and Hypusine-containing Dipeptides

Due to the fact that in recent studies hypusine has been routinely isolated using methods such as acid hydrolysis of proteins (Bergeron et al., 1998b), there is concern that its original isolation from bovine brains using trichloroacetic acid extracts may not have yielded free hypusine at all, but simply hypusine cleaved from eIF-5A. Complicating the matter further, the same group that performed the original isolation of hypusine has also reported the isolation of two hypusine-containing dipeptides: α-(γ-aminobutyryl)-hypusine and α-(β-alanyl)-hypusine (Sano et al., 1986, 1987; Ueno et al., 1991).

In order to explore this matter further, certain tools are required. The main tool required is a hypusine standard. A reliable, reproducible synthetic scheme is essential to access such a standard. The versatility of the hypusine reagent scheme developed by Bergeron, and described in detail above, was capitalized upon in order to prepare hypusine and the two hypusine-containing dipeptides—α-(β-alanyl)-hypusine and α-(γ-aminobutyryl)-hypusine (Fig. 2-2).

Hypusine. (2*S*, 9*R*) Hypusine was prepared according to the scheme described for the preparation of the hypusine reagent in Figure 2-1. Compounds **1**-**7** were synthesized according to the method of Bergeron (1998a) described above. Once the nitrile was reduced to give compound **6**, TFA and triisopropylsilane (TIS) were added to remove the BOC and *t*-butyl ester protecting groups. The compound was then purified by ion exchange chromatography to yield hypusine as the dihydrochloride salt.

The Dipeptides. Compounds **1**-**7** were synthesized according to the method of Bergeron (1998a) described above. Using a method modified from a procedure described by Gibson (1994), compound **12** was prepared by the selective removal of a BOC

protecting group in the presence of CBZ and *t*-butyl ester protecting groups (Fig. 2-2). First, hydrochloride gas was carefully bubbled into dry ethyl acetate for approximately 30 minutes followed by dilution with additional dry ethyl acetate to produce approximately 1 M HCl in ethyl acetate. (A freshly opened bottle of analytical grade ethyl acetate was used; ethyl acetate was further dried by eluting through a small column of alumina directly into the flask where HCl gas was introduced). The di-CBZ, BOC, *O*-*t*-butyl protected hypusine reagent (**7**) was then dissolved in the freshly prepared 1 M HCl in ethyl acetate and stirred at room temperature under nitrogen. The appearance of the free amine was monitored by TLC (both UV and ninhydrin development, 9:1 $CHCl_3$/MeOH). Compound **12** was obtained in 64% yield. Approximately 80% yields were obtained when compounds **13** and **14** were synthesized by coupling BOC-β-alanine-OH and BOC-γ-aminobutyric acid to compound **12** using the PyBOP® coupling reagent (Fig. 3-5) in methylene chloride (Coste et al., 1990). The coupling reaction was also monitored by TLC for the disappearance of starting materials and the emergence of fully protected dipeptide (1:5 C_6H_6/EtOAc). PyBOP® was utilized due to its description as a "safe, non-toxic alternative" to the widely used BOP reagent (Coste et al., 1990). Compounds **13** and **14** were individually deprotected via the addition of 30% HBr/HOAc and trifluoroacetic acid in the presence of pentamethylbenzene and phenol as cation scavengers. Extraction of the scavengers with methyl-*t*-butyl ether followed by concentration of the aqueous acetic acid layer yielded **15** and **16** (crude) as a mixture of trifluoroacetic acid and acetic acid salts. These compounds were converted to hydrochloride salts by eluting through an ion-exchange column with 0.1 to 6 N HCl. The collected fractions were then pooled and concentrated to yield **15** and **16** as hydrochloride

salts (clear, light yellow oils, >90% yields). (^{1}H and ^{13}C NMR spectra, optical rotation, HRMS data were obtained for compounds **12-16**). R_f values on TLC (Fig. 2-5a) agreed quite well with those presented in previous reports (Sano et al., 1986, 1987; Ueno et al., 1991). NMR data for the dipeptides will be presented and discussed in Chapters 4 and 6. These dipeptides, along with their hypusine precursor, were then utilized as standards for the development of an HPLC and LCMS analytical assay (Chapter 5).

Synthesis of ^{13}C-labeled and ^{3}H-labeled Hypusine Reagent

The hypusine reagent synthetic scheme is versatile enough to also allow various labels to be incorporated into the hypusine molecule. This is of great importance as the presence of a label would allow a researcher to follow the hypusine compound in various tracer and metabolism studies as well as enhanced NMR experiments.

^{13}C-labeled Hypusine Reagent. The formation of the hypusine reagent requires the addition of KCN and 18-crown-6 in order to integrate an additional carbon and terminal amine to the hypusine backbone (compound **5**, Fig. 2-1). Quite simply, commercially available K^{13}CN (Sigma-Aldrich) can be substituted into this synthesis (Fig. 2-3) and the ^{13}C-labeled hypusine reagent can be prepared as described above (**5a–10a**). The uniform incorporation of this label can be easily assessed by ^{13}C-NMR. These NMR experiments will be discussed in greater detail in Chapter 6. A portion of the ^{13}C-labeled hypusine reagent described here was utilized in the solid phase peptide synthesis of ^{13}C hypusine-containing eIF-5A peptide fragments discussed below (Chapter 3).

^{3}H-labeled Hypusine. A radioactive label can also be incorporated into the hypusine reagent synthetic scheme. The incorporation of nitrile functionality into the hypusine molecule was subsequently followed by a reduction with H_2 (g) in the presence of mixed catalysts Pd-C and PtO_2 in acetic acid to give **6** (Fig. 2-1). Alternatively, $^3H_2$

gas can be used to reduce the nitrile to a terminal amine and in the process incorporate two non-exchangeable tritium atoms at the C-11 position (Fig. 2-4).

A portion of compound **5** was reduced with $^3H_2$ (g) in the presence of mixed catalysts, Pd-C and PtO_2 in acetic acid, at Moravek Biochemicals (Brea, CA). As a result, compound **6b** with a ^3H label on C-11 was obtained (approximately 5 mL of 1.0 mCi/mL; 649.2 mg/mL in ethanol). Radiochemical purity as determined by TLC (1:4:4 $NH_4OH/CH_2Cl_2/MeOH$) was 98% and specific activity was 600 mCi/mmol. In Bergeron's laboratories, compound **6** was prepared by reduction with non-radioactive H_2 (g) and mixed catalysts PtO_2 and Pd-C in acetic acid (slightly yellow oil, ~68 % yield).

Due to the elevated level of radioactivity of the hypusine precursor, 50 mg of the unlabeled compound **6** was added to 1.5 mL of the radiolabeled **6b**. This "cold dilution" process resulted in a specific activity of 16 mCi/mmol (almost a 38 fold dilution from the stock solution). The solution containing both radiolabeled and unlabeled compound **6/6b** was concentrated via rotary evaporation followed by azeotropic removal of excess ethanol with methylene chloride. Then, additional methylene chloride was added and the reaction flask was cooled in an ice bath. TFA was added to the flask and the mixture was allowed to stir under N_2 (g) atmosphere for approximately one hour at room temperature. The mixture was again concentrated to dryness and then cooled in an ice bath. TFA was again added and the solution was allowed to stir at room temperature under a N_2 (g) atmosphere for approximately three hours. TLC analysis of the reaction mixture was used to determine when all starting material had been fully deprotected (2:1:1 $MeOH/CH_2Cl_2/NH_4OH$). Again, the contents of the flask were concentrated to dryness followed by methylene chloride washes. The radioactive residue was then carefully

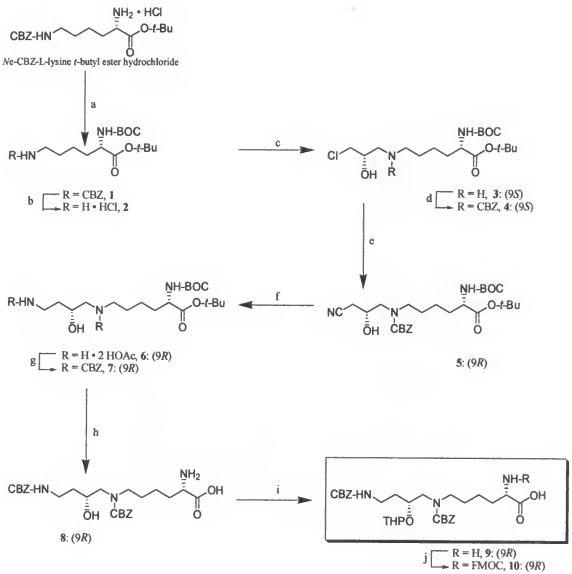
purified via flash chromatography (15 x 2.5 cm, flash silica, 2:1:1 methanol, methylene chloride, ammonium hydroxide).

As compound **18** eluted from the column, fractions were collected and analyzed by thin layer chromatography and scintillation counting. In this manner, the radiolabeled product was observed to co-elute with the unlabeled product. Figure 2-5b depicts how the scintillation counts complimented TLC analysis of the fractions collected. The strongest levels of radioactivity (CPM) were detected in the same fractions containing the compound with an R_f identical to that of the hypusine standard. Fractions 16-45 were transferred to a tared flask and concentrated to dryness followed by three 15 mL additions of methylene chloride to remove any excess methanol and ammonia. The product, a colorless oil, was then acidified via microliter additions of 0.1 N HCl (aq), transferred in 1 mL portions to small, brown glass vials, and stored in the freezer. A ^{3}H-labeled hypusine reagent of the same form as compound **10** has not yet been prepared. However, it is clear that the syntheses described here could easily be adapted to obtain a radiolabeled hypusine reagent. The ^{3}H-labeled hypusine compound was used as a tracer compound for the development of tissue extraction assays described in Chapter 5.

Preparation of Deoxyhypusine Reagent

The preparation of the deoxyhypusine reagent by Bergeron et al. (1998a) followed a scheme analogous to that discussed above for the hypusine reagent. Of course, the only difference in the structure of the final reagent is the lack of the hydroxyl group at C-9. First, following the method of Sumimoto, the N$^{\alpha}$-BOC-*tert*-butyl ester of L-lysine is reacted with 3-cyanopropanal in benzene over molecular sieves as shown in Figure 2-6 (Sumimoto & Kobayashi, 1966). This condensation reaction was followed by a reduction

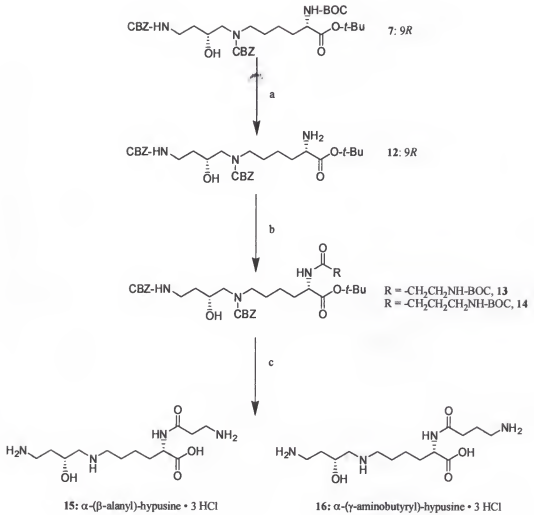
with H_2 (g) and PtO_2 catalyst to obtain the deoxyhypusine backbone. An additional reduction using H_2 (g) and a mixed catalyst (Pd-C/PtO_2) in acetic acid was required to convert the terminal nitrile to a terminal amine. The N-7 and N-12 amines were then protected via reaction with CBZ-ONSu, as for the hypusine reagent, to form the di-CBZ, BOC, *O-t*-butyl protected deoxyhypusine reagent. To allow this reagent to be incorporated into solid phase peptide synthesis, TFA and TIS were added to remove the BOC and *t*-butyl protecting groups. This was followed by the addition of FMOC-ONSu to protect the alpha nitrogen atom. A portion of the deoxyhypusine reagent (**19**) described here was utilized in the solid phase peptide synthesis of deoxyhypusine-containing eIF-5A peptide fragments discussed in Chapter 3.



Reagents: (a) $(BOC)_2O$, $NaHCO_3$; (b) H_2, Pd-C; (c) (S)-(+)- epichlorohydrin; (d) benzyl chloroformate, triethylamine; (e) KCN, 18-crown-6; (f) H_2, Pd-C, PtO_2; (g) CBZ-ONSu, $KHCO_3$; (h) TFA, triethylsilane, CH_2Cl_2; (i) 3,4-dihydro-2H-pyran; (j) FMOC-ONSu, Na_2CO_3

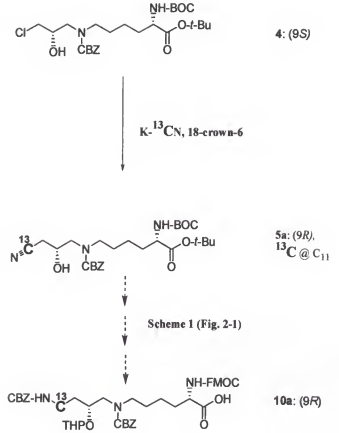
Bergeron et al., 1998a

Figure 2-1. Synthetic Scheme #1–Synthesis of Hypusine Reagent



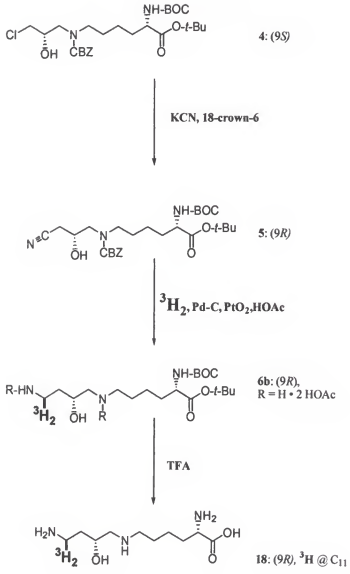
Reagents: (a) 1M HCl in ethyl acetate; (b) PyBOP, BOC-β-alanine or BOC-GABA, diisopropylethylamine, CH_2Cl_2; (c) 30% HBr/Acetic acid, trifluoroacetic acid, phenol, pentamethylbenzene followed by ion exchange w/ 0.1 to 6 N HCl

Figure 2-2. Synthetic Scheme #2–Synthesis of Hypusine Dipeptides



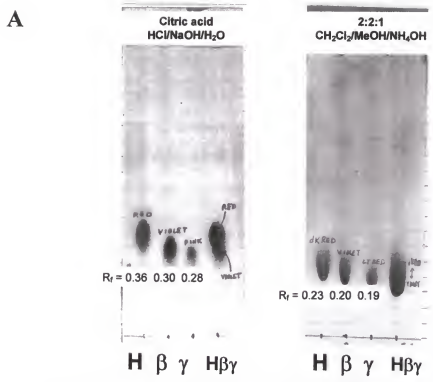
^{13}C labeled potassium cyanide is used to obtain the ^{13}C hypusine reagent

Figure 2-3. Synthetic Scheme #3–Synthesis of ^{13}C Hypusine Reagent



$^3H_2$ gas is used for the mixed catalyst reduction of the nitrile

Figure 2-4. Synthetic Scheme #4–Synthesis of ^{3}H-Hypusine



H: Hypusine, β: β-Ala-hypusine, γ: GABA-hypusine, Hβγ: co-spot

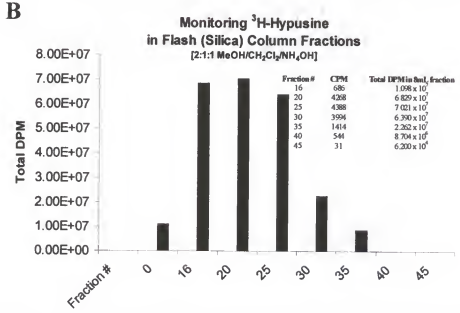
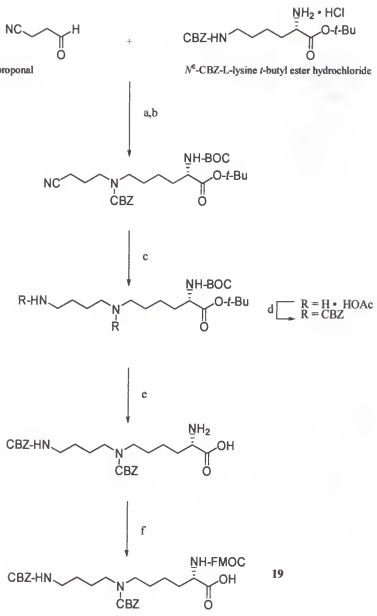


Figure 2-5. Analysis of Compounds During Synthesis-A) TLC for Hypusine Dipeptides and B) Scintillation Counts for ^{3}H-Hypusine



Reagents: (a) benzene, molecular sieves; (b) H_2, PtO_2, THF; (c) H_2, Pd-C, PtO_2; (d) CBZ-ONSu, $KHCO_3$; (e) TFA, triethylsilane, CH_2Cl_2; (f) FMOC-ONSu, Na_2CO_3

Bergeron et al., 1998a

Figure 2-6. Synthetic Scheme #5–Synthesis of Deoxyhypusine Reagent

CHAPTER 3
SYNTHESIS II–SOLID PHASE

In order to more completely understand the mechanisms by which hypusine is formed on eIF-5A and the roles this amino acid and protein play in nature, it is key to develop routine access to these compounds synthetically. The synthesis of hypusine, hypusine dipeptides, and various labeled hypusine compounds has been described in Chapter 2. This chapter focuses on solid phase peptide synthetic techniques and the incorporation of the hypusine, deoxyhypusine, and ^{13}C-hypusine reagents into peptide fragments matching the sequence of the eIF-5A protein surrounding the Lys_{50}-Hyp_{50} modification site (Figures 1-5, 3-7, and 3-8).

The synthesis of hypusine-containing peptides up to 21 amino acids in length is described below. The successful preparation of such peptides demonstrates the utility of the various hypusine reagents. To date, the hypusine and deoxyhypusine reagents have only been incorporated into hexapeptides (Bergeron et al., 1998a). The ^{13}C-hypusine reagent was previously untested. The successful incorporation of these reagents into larger peptides via solid phase peptide synthesis described below displays the potential for preparing the entire eIF-5A protein synthetically in its three naturally occurring stages: $Lysine_{50}$ (eIF-5A precursor), $Deoxyhypusine_{50}$ (eIF-5A intermediate), and $Hypusine_{50}$ (mature, fully functional eIF-5A). The availability of such proteins would provide the necessary substrates for which to study the two key enzymes known to be responsible for the post-translation modification of eIF-5A: deoxyhypusine synthase (DOHS) and deoxyhypusine hydroxylase (DOHH). Furthermore, analysis of the 20

amino acid peptide fragments by various techniques such as high field NMR may help to define the hypusine modification site more fully and provide an NMR fingerprint of this post-translational modification site as well. This information is advantageous for future experiments that would attempt to observe the interactions of these peptides with other viral proteins such as Rev and the Rev Response Element in HIV.

Solid Phase Peptide Chemistry (SPPS)–General

In 1963, Merrifield introduced solid phase peptide synthesis (SPPS). His idea was to develop methodologies that would enhance the field of peptide chemistry and facilitate the synthesis of previously unattainable compounds. SPPS demonstrated the first practical use of an insoluble polymer with bound reagents in organic synthesis (Stewart & Young, 1984). Merrifield linked the C-terminal residue of the peptide to be synthesized to an insoluble polymer. Through a series of deprotection and coupling steps, the peptide was synthesized one residue at a time from the C- to N-terminus. Numerous papers have been published using SPPS as the means to synthesize a variety of peptides of varying lengths. Solid phase peptide synthesis has been reviewed extensively elsewhere (Stewart & Young, 1984; Fields & Noble, 1990; Chan & White, 2000; Pennington & Dunn, 1994). This paper will focus on the use of Fmoc SPPS only.

Fmoc SPPS refers to the use of $N\alpha$-Fmoc protected amino acids during the synthesis of the peptide. The Fmoc group is labile to mildly basic conditions (i.e. 20% piperidine in DMF) whereas the side-chain protecting groups are typically labile to acidic conditions (i.e. TFA) as is the resin linker. The hypusine and deoxyhypusine reagents discussed earlier are well suited for use in Fmoc SPPS and their incorporation into eIF-5A peptide fragments is discussed below.

Reaction Vessel

The reaction vessel used for SPPS is a glass cylinder that contains a glass frit at the bottom leading to a three-way valve (Fig. 3-1). This valve allows for the introduction of an inert gas such as N_2 from beneath the frit to allow gentle mixing of the resins and reagents when in the vessel. When reactions are being stirred by the gas flow, they are said to be "bubbling". When reactions have gone to completion, excess reagents and solvents are drained through the frit into a waste container leaving the peptide bound to the resin inside the reaction vessel. The unique design allows SPPS to be a "one-pot" method.

Prior to the synthesis of the various peptides described below, the reaction vessels were acid washed and silanized. This is a necessary step prior to SPPS because many peptides, especially those that exhibit basic and hydrophobic characteristics, adhere to the surfaces of the glassware. Silanization of the glass reaction vessel prevents this from occurring and facilitates the SPPS procedure (Stewart &Young, 1984).

Silanization. First, concentrated H_2SO_4 was added to the reaction vessel and allowed to "bubble" with N_2 for at least one hour. The acid was then drained and the reaction vessel was rinsed with copious amounts of distilled water. The vessel was then dried in an oven. After drying and returning to room temperature, 10% dichlorodimethylsilane in dry toluene was added to the reaction vessel and allowed to bubble with N_2 for one hour. After draining the silanization solution, the reaction vessel was immediately rinsed three times with excess dry toluene. Following this, the vessel was filled with dry methanol and allowed to bubble with N_2 for 15 minutes. The

methanol was then drained and the vessel was rinsed with copious amounts of methanol (three washes) followed by acetone (three washes). The reaction vessel was then thoroughly dried in an oven overnight (personal communication, Dr. Haskell-Luevano, University of Florida).

FMOC Chemistry and Wang Resin

The hypusine reagents were designed for use in standard Fmoc solid phase peptide synthesis. Thus, it was necessary to choose an Fmoc-amino acid substituted resin suitable for this type of chemistry so that these novel reagents could be incorporated into larger peptides. Wang resin, considered one of the standard supports used in Fmoc SPPS, was developed by Wang in 1973 and was chosen for the syntheses of all peptide acids described in this dissertation (Fig. 3-2). The resin consists of 1% divinylbenzene beads, 100-200 mesh, onto which an acid-labile linker, *p*-hydroxybenzyl alcohol has been attached (Wang, 1973). Wang resin was originally prepared by allowing Merrifield's resin to react with either methyl-4-hydroxybenzoate and $NaOCH_3$ followed by $LiAlH_4$ reduction or 4-hydroxybenzyl alcohol and $NaOCH_3$ to form *p*-hydroxybenzyl alcohol substituted resin. The *p*-hydroxybenzyl alcohol linker portion of the resin can then be attached to the first N^{α}-Fmoc protected amino acid in the desired peptide sequence (Wang, 1973).

Fmoc-Leu-Wang resin. In order to synthesize peptides containing the amino acid sequence surrounding the Lys_{50} residue for post-translational modification to hypusine in eIF-5A, Fmoc-Leu-Wang Resin (Leu_{58} in the human eIF-5A peptide sequence) with a 0.36 mmol/g substitution (1.0 g resin = 0.127 g FMOC-Leu + 0.873 g resin) was purchased from Peptides International (Fig. 3-2). Beginning with Leu_{58}

allowed the syntheses of peptides containing eight and up to 12 amino acids respectively on either side of the Lys_{50} modification site. Higher resin efficiency was available for purchase but was avoided due to earlier experiences of peptide aggregation, difficult couplings, and cross-linking known to occur during solid phase peptide synthesis at higher resin substitutions (Pennington & Dunn, 1994).

The powder-like FMOC-Leu-Wang resin (1.0 g) was placed into the reaction vessel and solvated or "swelled" in 20 mL of dry distilled DMF with N_2 bubbling for at least three hours prior to the first deprotection and coupling cycle. A worksheet was prepared so as to standardize each step and minimize errors in the SPPS method (Stewart & Young, 1984; Haskell-Luevano, personal communication).

FMOC-deprotection

The 9-fluorenylmethoxycarbonyl (Fmoc) protecting group developed by Carpino and Han (1970, 1972) was designed to mask primary amines, rendering them inactive as nucleophiles. This protecting group is desirable in a multi-step synthesis as well as SPPS because it exhibits acid stability. Other protecting groups such as BOC and benzyl-based groups can be cleaved in its presence thus adding Fmoc to the list of orthogonal protecting groups. Only under basic conditions can the Fmoc be cleaved to reveal a free, terminal amine (Greene & Wuts, 1999).

The fluorene ring system of the Fmoc protecting group exhibits an electron withdrawing effect on the hydrogen on the β-carbon. This causes the hydrogen to be acidic and therefore readily removed by a base such a piperidine (pKa 11.22) (Carpino & Han, 1970). The products of this reaction are a DMF-soluble dibenzofulvene (which may form an adduct with piperidine), carbon dioxide, and the free amine (Fig. 3-3a). The

absence of acid in this reaction ensures that the resultant amine is indeed free (Bodansky, 1993). Other deprotection methods, such as the use of trifluoroacetic acid for the removal of BOC protecting groups, generate a protonated amine. This protonated amine must then be converted to the free amine prior to coupling. Deprotection of the Fmoc group with a base such as piperidine, and followed by simply rinsing the resin with excess DMF, generates the free amine directly making it immediately available for peptide coupling.

In this laboratory, the Fmoc protecting group was removed from the amino terminus of the resin-bound peptide in the following manner. After the resin was properly swelled in DMF, a solution of 20-30% piperidine in DMF was added to the resin first for two minutes with N_2 agitation. The solution was drained and the procedure was repeated with fresh 20-30% piperidine in DMF for 25 min. The $t_{1/2}$ for the deprotection of Fmoc-Valine-OH with 20% Piperidine in DMF is six seconds—excess reaction times ensured complete removal of the Fmoc protecting group (Greene & Wutz, 1999). The resin was then washed four times with DMF to remove any traces of piperidine. A small sample of the resin was then removed from the batch and subjected to the Kaiser (Ninhydrin) test (discussed later in this chapter). This test determines if the Fmoc protecting group has been removed to reveal a terminal -NH_2. This free amine is available to act as a nucleophile in the coupling of the next amino acid in the peptide sequence.

Amino Acid Activation and Coupling

The mechanism for the activation and coupling of amino acids during solid phase peptide synthesis is detailed in Figure 3-3b and Figure 3-4. Fmoc SPPS typically

requires the formation of benzotriazole esters on the amino acids to be coupled (Fields & Noble, 1990). Activation agents used for this procedure are (1) benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate (BOP), (2) benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), (3) *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), and (4) 1-hydroxybenzotriazole (HOBt) (Fig. 3-5).

After the resin is properly solvated in DMF and the terminal Fmoc protecting group is removed, the next Fmoc-protected amino acid is dissolved in a solution of DMF and activating agents to produce a benzotriazole ester on its carboxyl terminus (Fmoc amino acid reagents are shown in Fig. 3-6). This solution is then added to the deprotected peptide-resin in the reaction vessel and allowed to bubble with N_2. Reactions times are typically 2–3 hours. Some couplings required the filtration and re-addition of coupling reagents and occasionally overnight reactions times to obtain complete couplings. After the reaction is determined to be complete by the Kaiser test, excess reagents are drained away leaving the now elongated, resin-bound, terminally Fmoc protected peptide in the reaction vessel. Coupling reactions are monitored as described below. It should be noted that nucleophilic sites on the side chains of all amino acids used during the peptide synthesis are blocked with protecting groups that are stable to the conditions of the deprotection and coupling cycle.

Kaiser (Ninhydrin) Test

It is necessary to monitor the SPPS reaction at each coupling step in order to avoid incomplete peptide chains, or deletion sequences, from developing throughout the

synthesis. Kaiser et al. first reported a method for the determination of the completeness of amino acid coupling during SPPS in 1970. Peptide chemists routinely use this method today. Based on the ninhydrin reagent described by Troll and Canan (1953), the ninhydrin reagent of the Kaiser test reacts with free $N\alpha$-amino groups to form a deep blue/violet color known as Ruhemann's purple (λ_{max} 570 nm).

Kaiser test reagent. Three solutions were prepared for the Kaiser test as follows: (1) 2 mL of 0.001 M KCN (6.5 mg KCN in 100 mL distilled, deionized H_2O) diluted to 100 mL with pyridine; (2) 50 mg ninhydrin (Aldrich) in 1 mL ethanol; (3) 80 g phenol in 20 mL ethanol. After a coupling reaction or Fmoc deprotection, a small sample of the resin was removed from the reaction vessel on a pipette tip and placed in a glass test tube. Approximately 20-30 µL of each reagent were added to the resin. The solution was mixed via mild shaking or vortexing for five seconds and then heated in a heating block (110 °C) for four minutes. A clear, golden yellow solution color identical to that of ninhydrin in ethanol was indicative of a negative result (i.e. no free amines) or complete coupling. In strong contrast, the presence of free $N\alpha$-amines after Fmoc deprotection or an incomplete coupling reaction caused the solution to turn deep blue/violet.

It should be noted that the Kaiser test, as described above, is only semi-quantitative. The amount of free amine present is estimated visually based on the intensity of the blue/violet color observed in the test solution. Stated more simply, the darker the blue/violet color, the more free amine is considered to be present (Sarin et al., 1981). Kaiser described peptide couplings (verified by titration) ranging from 76% to 99.4% completion as exhibiting "dark blue to trace blue solution" colors respectively (Kaiser et al., 1970). As coupling proceeds to completion, the terminal $N\alpha$-amines

become amides in the peptide backbone and therefore are unavailable to react with the ninhydrin reagent. When only a clear golden yellow color is observed, the coupling reaction is said to be complete.

Despite the Kaiser test's visual indication that a coupling is complete, it is possible that a small percentage of free amine may still be present on the resin. This minor amount of $N\alpha$-amine may not cause a color change significant enough to be observed by the naked eye. Unprotected, this amine is available to react in subsequent coupling cycles. Over the course of a lengthy peptide synthesis, this small percentage of uncoupled amine can lead to a significant amount of deletion peptide. A more accurate quantitative method for the monitoring of SPPS by the ninhydrin reaction was proposed by Sarin et al. (1981) and has more recently been standardized by various automated peptide synthesis equipment manufacturers. This method involves monitoring the ninhydrin chromophore (Ruhemann's purple) in solution at 570 nm. However, this method may be considered a slightly tedious and unnecessary measure to the manual synthetic peptide chemist. Since acylations/couplings are considered high yield reactions, very often these highly quantitative measures determine that only fractions of a percent of $N\alpha$-amines remain unreacted (Fields & Noble, 1990). Therefore, these methods are more practical when incorporated into an automated peptide synthesizer where the chemist cannot personally sample the beads from the reaction vessel after each deprotection and coupling cycle. For manual SPPS the practice of "capping", even when the visual Kaiser test indicates complete coupling, eliminates any unreacted amines that may remain on the resin. Capping, described below, is more commonly and easily

employed during manual SPPS and was used throughout the synthesis of the peptides described in this paper.

In summary, the Kaiser test was utilized to monitor the status of terminal amines on the resin-bound eIF-5A peptide fragments throughout each step of SPPS described in this dissertation. A coupling was determined to be incomplete if any color other than that of the ninhydrin reagent itself was observed after heating the resin in the presence of the ninhydrin reagents. Occasionally, when applying the Kaiser test to assess the extent of FMOC deprotection (prior to coupling), even the sampled resin beads were stained. This staining of the beads has been attributed to the blue/violet chromophore being ionically bound to the resin bearing a positive charge (Sarin et al., 1981).

Capping

Deletion peptides that differ from the desired peptide sequence by only one or two residues may not be easily separated from the desired peptide by HPLC or other purification techniques. For example, their affinity for the HPLC column is nearly identical causing them to coelute. Further, mass spectrometry and Edman degradation (sequence) analysis of what appears to be a single, pure compound by HPLC usually reveals that there is indeed more than a single peptide sequence present. Therefore, terminating the peptide chain by "capping" any unreacted *N*α-amines prior to the next coupling substitutes a terminated peptide for a potential deletion peptide. Theoretically, terminated peptides will then differ from the desired peptide significantly enough, for example in both molecular weight and N-terminal functionality (*N*-acetyl-peptide vs. NH_2-peptide), that separation by HPLC methods should be relatively simple (Fields & Noble, 1990; Dunn & Pennington, 1994).

Capping reagent. During the synthesis of the peptides described in this paper, any uncoupled amines were blocked using acetic anhydride in the presence of a tertiary amine such as DIEA in CH_2Cl_2 (Stewart & Young, 1984; Pennington & Dunn, 1994). This reactive agent effectively acetylated any primary amines still available on the peptide-resin. Specifically, a 2:1:2 ratio of acetic anhydride/DIEA/CH_2Cl_2 was prepared. After each coupling was deemed complete by the traditional Kaiser test, 10-20 mL of this "capping" solution were added to the resin and allowed to bubble with N_2 for 15-30 minutes. The solution was then drained and the resin was rinsed with excess CH_2Cl_2 three times, followed by five rinses with DMF. All steps were recorded on the peptide worksheet as described earlier. In this manner, the amino termini of any peptide chains where coupling may have been incomplete were covalently blocked (Fields & Noble, 1990). Facilitating the purification process by terminating unwanted peptide chains prior to cleaving the peptide from the resin enhanced the final yields of the desired peptide sequences.

Synthesis of eIF-5A Peptide Fragments

The sequences and expanded structure of the peptides synthesized in this study using the Fmoc SPPS methods described above are shown in Figures 3-7 and 3-8. Peptides of 5, 8, 14, and 20 amino acid residues of the human eIF-5A sequence have been synthesized from Leu_{58} toward Lys_{39}. These peptides contain a lysine residue at position 50 and represent the unmodified form of eIF-5A. In addition, 20-residue sequences containing deoxyhypusine, hypusine, and ^{13}C-labeled hypusine at position 50 have also been synthesized using the reagents described in the previous chapter. A 21-residue sequence, Leu_{58} to Cys_{39}, with hypusine at position 50 was also prepared. To determine their purity and sequence integrity, each peptide was carefully characterized by HPLC,

MALDITOF-MS, and amino acid sequencing. The peptides have also been analyzed by nuclear magnetic resonance at various temperatures, pHs, and field strengths (Chapter 6).

A typical peptide synthesis followed a series of deprotection and coupling steps as depicted in Figure 3-1. Prior to each reaction, the resin was swelled for 1-2 minutes in DMF and rinsed with excess DMF. The terminal Fmoc was removed via a two minute then 25 minute reaction with 20-30% piperidine in DMF. This was followed by four washes with excess DMF. A portion of the resin was sampled and subjected to the Kaiser test. If a positive result was obtained, the next amino acid in the peptide sequence and the appropriate activating agents (i.e. HBTU, HOBT, etc.) were dissolved in DMF and added to the reaction vessel with DIEA. Typical coupling times were 2-3 hours. Occasionally a reaction was observed to be incomplete after this time period. Reagents were then drained from the reaction vessel and fresh reagents were added and allowed to react overnight. Upon completion of coupling (as determined by the Kaiser test), the resin was washed with excess DMF and CH_2Cl_2 followed by the addition of "capping" reagents to terminate any unreacted peptide chains that might remain on the resin. After capping, the resin was again washed with excess DMF and the cycle of deprotection and coupling was repeated.

Final Deprotection and Cleavage from Resin

After the successful coupling of the final amino acid in the peptide sequence, the terminal Fmoc was removed as described above. The peptide was then subjected to chemical conditions that simultaneously removed all side chain protecting groups and cleaved the peptide from the solid support. A cleavage "cocktail" was prepared with 95% trifluoroacetic acid, 2.5% H_2O, and 2.5% triisopropylsilane (as scavenger). An

excess of the cleavage cocktail was added to the resin and mixed with a gentle N_2 gas flow. After 2.5 hours, the solution was drained and collected. The resin was washed three times with excess cleavage cocktail. All washes were collected and then partitioned into centrifuge tubes. Cold ether was added to each tube in order to precipitate the peptide and extract scavengers and side chain protecting groups. The tubes were sealed and centrifuged for 15 min at 2500 rpm, 10 °C. The ether was carefully decanted leaving a white residue in the centrifuge tubes. A total of three cold ether extractions were carried out. The peptide residues were allowed to dry in a desiccator under vacuum overnight.

Peptides prepared with Hypusine reagents. Peptides containing the deoxyhypusine, hypusine, and ^{13}C-labeled hypusine reagents required the use of a slightly different cleavage cocktail due to the presence of CBZ protecting groups that were not labile to trifluoroacetic acid cleavage conditions. For these peptides, the resin-bound peptide was first transferred from the reaction vessel to a round bottom flask and then cooled in an ice bath. A cleavage cocktail consisting of 10 mL TFA, 500 μL of TIS, phenol, and pentamethylbenzene (also cooled) was then added. The mixture was stirred for five minutes under N_2 followed by the addition of 400 μL of 30% HBr in acetic acid under N_2 for 15 minutes. The flask was allowed to warm to room temperature. After 1.5 hours, the resin beads were filtered away and the solution was concentrated to near dryness via rotary evaporation. The residue was then treated with 10% acetic acid (aq) and extracted with cold diethylether. The aqueous layer was then lyophilized. All crude peptides were purified via preparative HPLC (Chapter 4).

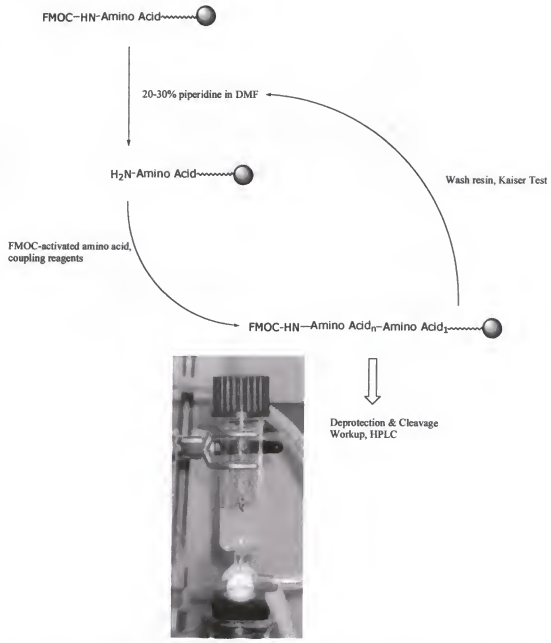
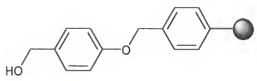
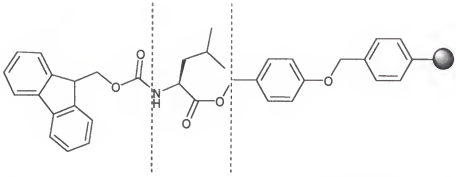


Figure 3-1. General FMOC Solid Phase Peptide Synthesis and Reaction Vessel

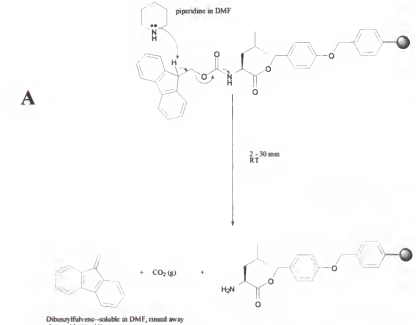


p-Alkoxylbenzylalcohol (WANG RESIN)

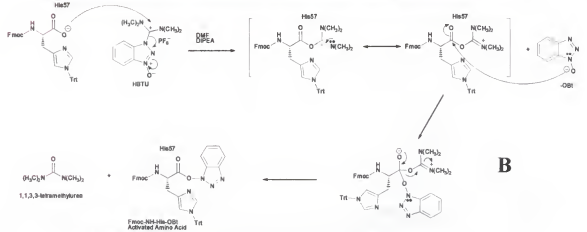


FMOC-protecting group *Leucine*$_{58}$ *p*-Alkoxylbenzylalcohol (WANG RESIN)

Figure 3-2. FMOC-Leu$_{58}$-WANG RESIN

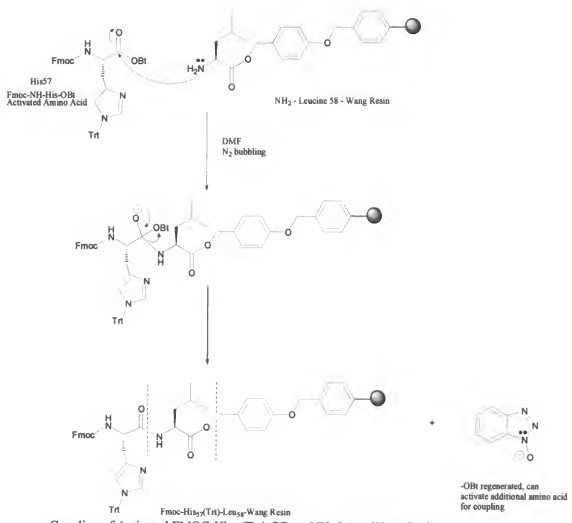


Deprotection of FMOC-Leu_{58}-Wang Resin with 20-30% Piperidine in DMF



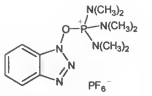
Activation of FMOC-His_{57}(Trt)-OH with HBTU/DIEA/DMF

Figure 3-3. FMOC Synthesis–A) Deprotection and B) Activation of FMOC Amino Acids for SPPS



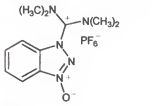
Coupling of Activated FMOC-His_{57}(Trt)-OBt to NH_2-Leu_{58}-Wang Resin

Figure 3-4. Amino Acid Coupling in SPPS



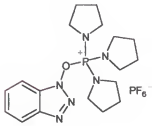
$C_{12}H_{22}F_6N_5OP_2$
Mol. Wt.: 442.28

BOP



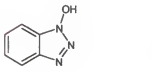
$C_{11}H_{16}F_6N_5OP$
Mol. Wt.: 379.24

HBTU



$C_{18}H_{28}F_6N_5OP_2$
Mol. Wt.: 520.39

PyBOP



$C_6H_5N_3O$
Mol. Wt.: 135.12

HOBt

Figure 3-5. Structures of Coupling Reagents used in SPPS

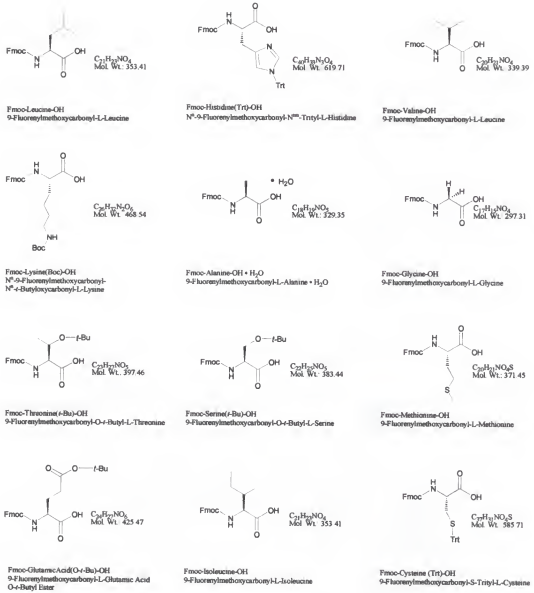


Figure 3-6. Structures of Standard FMOC-protected Amino Acids used in SPPS

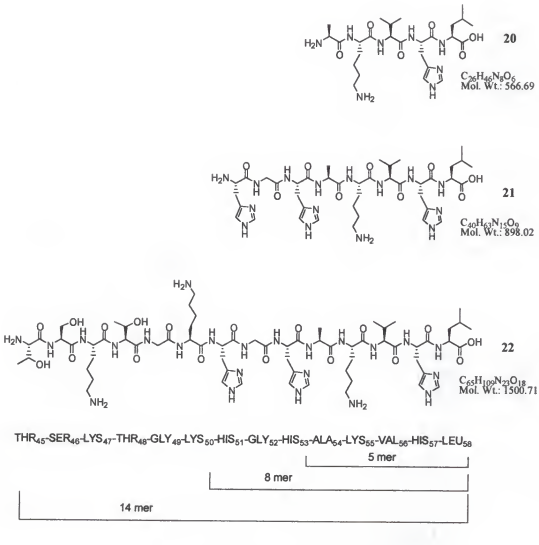
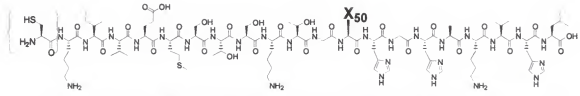


Figure 3-7. eIF-5A 5-, 8-, and 14-Amino Acid Peptide Fragments



(CYS$_{38}$)-LYS$_{37}$-ILE$_{40}$-VAL$_{41}$-GLU$_{42}$-MET$_{43}$-SER$_{44}$-THR$_{45}$-SER$_{46}$-LYS$_{47}$-THR$_{48}$-GLY$_{49}$-**X$_{50}$**-HIS$_{51}$-GLY$_{52}$-HIS$_{53}$-ALA$_{54}$-LYS$_{55}$-VAL$_{56}$-HIS$_{57}$-LEU$_{58}$

X_{50} = Lysine — **23**

$C_{95}H_{162}N_{30}O_{27}S$
Mol. Wt.: 2188.55

X_{50} = Deoxyhypusine — **24**

$C_{99}H_{171}N_{31}O_{27}S$
Mol. Wt.: 2259.68

X_{50} = Hypusine — **25**

$C_{99}H_{171}N_{31}O_{28}S$
Mol. Wt.: 2275.68

X_{50} = ^{13}C-Hypusine — **26**

$C_{98}{}^{13}CH_{171}N_{31}O_{28}S$
Mol. Wt.: 2276.68

X_{50} = Hypusine, (Cysteine $_{38}$) — **27**

$C_{102}H_{176}N_{32}O_{29}S_2$
Mol. Wt.: 2378.82

Figure 3-8. eIF-5A 20 and 21 Amino Acid Peptide Fragments with Lys$_{50}$, DHyp$_{50}$, Hyp$_{50}$, and ^{13}C Hyp$_{50}$

CHAPTER 4
EXPERIMENTAL-SYNTHESIS I AND II

General

Reagents were purchased from various chemical companies such as Aldrich, Fluka, or Sigma and were used without further purification. *Nε*-CBZ-L-lysine *tert*-butyl ester hydrochloride was obtained from BACHEM Bioscience Inc. BOC-β-alanine-OH and BOC-γ-aminobutyric acid were purchased from NovaBiochem and PyBOP® coupling reagent was purchased from Acros. Fisher Optima-grade solvents were used routinely and organic extracts were dried with anhydrous Na_2SO_4. Acetonitrile used for the KCN/18-crown-6 reaction was distilled from NaH. HCl in ethyl acetate (1 M) was prepared by bubbling HCl (g) into dry ethyl acetate and then diluting to 1 M with additional dry ethyl acetate. Ethyl acetate was dried by elution through Al_2O_3 into a clean, dry flask under N_2 atmosphere. KCN was finely and carefully ground and dried for 20 h at 145 °C. Silica gel 32-60 (40 μm "flash") from Selecto, Inc. (Kennesaw, GA) was used for flash column chromatography. Molecular biology grade AG 50W-X8 Resin (200-400 mesh, Na^+ form) from BioRad was used for ion exchange chromatography. ^1H NMR and ^{13}C NMR spectra were recorded on a 300 MHz (Varian) for hypusine and hypusine dipeptide precursor compounds. The 500 and 750 MHz (Avance Bruker, 5mm TXI or BBO probes) instruments at the UF McKnight Brain Institute Advanced Magnetic Resonance and Imaging Spectroscopy (AMRIS) Facility were used to analyze the eIF-5A peptide fragment compounds. Samples were analyzed at room temperature (~ 25 °C) and pH was not adjusted unless otherwise stated (as for pH titration analyses described in

Chapter 6). Solvent and reference standard conditions are stated for each compound analyzed. Chemical shifts for proton NMR are given in parts per million downfield from an internal reference standard such as tetramethylsilane (TMS), or external reference standard such as sodium 3-trimethylsilylpropionate (TSP) in D_2O. ^{13}C NMR chemical shifts in CD_3OD are calibrated on the CD_3OD septuplet peak at 49.0 ppm unless otherwise stated. ^{13}C NMR chemical shifts for eIF-5A peptides are referenced to external TSP in D_2O. Coupling constants (*J*) are in hertz. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. FAB mass spectra were run in a 3-nitrobenzylalcohol matrix. Elemental analyses were run at Atlantic Microlabs (Norcross, GA). Optical rotations were obtained at 589 nm (the Na D-line) on a Perkin Elmer 141 Polarimeter with *c* expressed as grams of compound per 100 mL. $^3H_2$ gas hydrogenation was performed at Moravek Biochemicals Inc. (Brea, CA). A Beckman LS 5000 TD was utilized for scintillation counting.

The hypusine reagents (**10**, **10a**, and **19**) were prepared as previously described. All other Fmoc-amino acids, reagents and reaction vessels for SPPS were purchased from Peptides International. Crude peptides were routinely purified by HPLC on a Vydac C_{18} "semi-prep" reverse phase column (25cm x 1cm). The Gilson HPLC system consisted of two model 306 pumps, a model 805 manometric module, a model 811C dynamic mixer, a model 119 UV/Vis detector, a 401 dilutor, and a model 231 sample injector. The flow rate was set to 5.00 mL/min with a 30 minute solvent gradient (10% CH_3CN, 0.1% TFA in H_2O to 100% CH_3CN). The detector was set to $\lambda = 220$ nm. An Isco "Foxy" fraction collector was used to collect purified peptides.

MALDITOF-MS was performed on a Perseptive Biosystems Voyager DE PRO Biospectrometry Workstation (Framingham, MA) at the UF ICBR Protein Chemistry Core Facility. Samples were run in an α-cyano-4-hydroxycinnamic acid matrix (10 mg α-cyano-4-hydroxycinnamic acid dissolved in 1 mL 1:1 0.1% TFA in H_2O and CH_3CN). Amino acid sequencing using standard Edman degradation chemistry was also performed at the UF ICBR Protein Chemistry Core Facility on an Applied Biosystems 494 HT Procise Protein Sequencer (Foster City, CA). Samples for amino acid analysis (AAA) were hydrolyzed with 6 N HCl and run on an Applied Biosystems 420 Amino Acid Analyzer (Milford, MA) with a Water Associates PicoTag Workstation for hydrolysis at the UF ICBR Protein Chemistry Core as well. Precolumn PTC-derivatization was employed with this method.

Synthesis of α-(β-alanyl)- and α-(γ-aminobutyryl)-Hypusine Precursors

***Nα*-BOC-*Nε*-CBZ-L-lysine *tert*-Butyl Ester (1).** Sodium hydrogen carbonate (2.81 g, 33.47 mmol) in water (75 mL) was added to N^{ε}-CBZ-L-lysine *tert*-butyl ester hydrochloride (12.00 g, 32.18 mmol) in 100 mL chloroform. The mixture was stirred at room temperature for 5 min under an N_2 atmosphere. Di-*tert*-butyl dicarbonate (7.02 g, 32.18 mmol) in chloroform (50 mL) was added and the mixture was refluxed for 2.0 h and then allowed to cool to room temperature. The layers were separated, the aqueous layer was extracted with chloroform (3 × 100 mL), and the combined organic layers were dried. The organic layers were concentrated to dryness and purified by flash chromatography (20% ethyl acetate/hexane) to yield **1** (14.04 g, 99.9%) as a colorless oil: ^{1}H NMR (CD_3OD, TMS): δ1.30-1.82 (m, 6 H), 1.43 (s, 9 H), 1.45 (s, 9 H), 3.11 (t, 2 H, J = 6.7), 3.93 (dd, 1 H, J = 8.2, 5.2), 5.06 (s, 2 H), 7.24-7.38 (m, 5 H); ^{13}C NMR: δ24.0,

28.3, 28.7, 30.4, 32.4, 41.4, 55.8, 67.3, 80.4, 82.5, 128.8, 128.9, 129.4, 138.4, 158.1, 158.9, 173.8; $[\alpha]^{21}_{D}$ +4.3° (*c* 1.75, $CHCl_3$).

***N*α-BOC-L-lysine *tert*-Butyl Ester Hydrochloride (2)**. Compound **1** (14.01 g, 32.09 mmol) was dissolved in ethanol (122 mL) and 1 N HCl (36 mL). A catalyst, 10% Pd-C (1.20 g), was added and H_2 gas was introduced. After 20.5 h the black suspension was filtered through Celite and washed with ethanol. The filtrate was concentrated, and the residue was dried in vacuo to give **2** as the hydrochloride salt (11.89 g, 100%): ^{1}H NMR (CD_3OD, TMS): δ 1.40-1.82 (m, 6 H), 1.44 (s, 9 H), 1.46 (s, 9 H), 2.92 (t, 2 H, *J* = 7.6), 3.95 (dd, 1 H, *J* = 8.9, 4.9); ^{13}C NMR: δ 23.9, 28.0, 28.3, 28.7, 32.1, 40.5, 55.5, 80.5, 82.7, 158.2, 173.5; $[\alpha]^{23}_{D}$ –20.0° (*c* 0.86, CH_3OH).

(2*S*,9*S*)-2-[(*tert*-Butoxycarbonyl)amino]-10-chloro-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (3). A solution of **2** (10.87 g, 32.09 mmol) in chloroform (200 mL) was extracted with saturated $NaHCO_3$ solution (2 × 150 mL) to neutralize the HCl salt. The aqueous layer was extracted with chloroform (4 x 100 mL). The organic layer was dried, concentrated, and further dried under vacuum. The resulting oil was dissolved in cyclohexane (60 mL). (*S*)-(+)-epichlorohydrin (3.25 g, 35.12 mmol) was added under an Ar atmosphere. After 35 h, the precipitated product was filtered, washed with cold cyclohexane, and dried under vacuum to give **3** (4.89 g, 39%) as a fine, white powder: The procedure described was repeated with an additional 3.35 g of **2** and yielded 1.28 g (33% yield) of the white powder. mp 86–88 °C; ^{1}H NMR (CD_3OD, TMS): δ 1.30-1.82 (m, 6 H), 1.43 (s, 9 H), 1.45 (s, 9 H), 2.58-2.65 (m, 3 H), 2.76 (dd, 1 H, *J* = 12.2, 3.8), 3.51 (dd, 1 H, *J* = 11.2, 5.7), 3.56 (dd, 1 H, *J* = 11.1, 5.0), 3.84-3.91 (m, 1 H), 3.94 (dd, 1

H, J = 9.1, 5.6); ^{13}C NMR: δ 24.6, 28.3, 28.7, 30.0, 32.6, 48.2, 50.3, 53.5, 55.8, 71.1, 80.4, 82.5, 158.1, 173.8; $[\alpha]^{23}_{D}$ –24.9° (*c* 1.00, CH_3OH).

(2*S*,9*S*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-chloro-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (4). A solution of benzyl chloroformate (3.67 g, 21.50 mmol) in chloroform (30 mL) was added over a period of 15 min to an ice-cold solution of **3** (6.05 g, 15.31 mmol) in chloroform (40 mL) under an Ar atmosphere. Triethylamine (3.09 g, 30.62 mmol) in chloroform (30 mL) was then added dropwise and the reaction mixture was stirred for 4.5 h at room temperature. The reaction mixture was extracted with 1 N HCl (160 mL) and water (160 mL). The aqueous extract was dried, concentrated and purified by flash chromatography (33% ethyl acetate/hexane) to yield **4** (6.89 g, 85%) as a colorless oil: ^{1}H NMR (CD_3OD, TMS): δ 1.30-1.82 (m, 6 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 3.21-3.55 (m, 6 H), 3.93 (dd, 1 H, J = 8.6, 5.5), 4.0 (m, 1 H), 5.12 (s, 2 H), 7.30-7.37 (m, 5 H); ^{13}C NMR: δ 24.1, 28.3, 28.8, 32.5, 49.4, 52.2, 55.7, 68.4, 71.1, 80.4, 82.5, 129.0, 129.1, 129.6, 138.0, 157.5-158.4 (br), 158.1, 173.7; $[\alpha]^{23}_{D}$ –21.2° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-7-(carbobenzyloxy)-10-cyano-9-hydroxy-7-azadecanoic Acid *tert*-Butyl Ester (5). A mixture of dry KCN (9.24 g, 141.89 mmol), 18-crown-6 (1.021g, 3.86 mmol), and **4** (6.81 g, 12.87 mmol) in dry acetonitrile (300 mL) was stirred at 60 °C for 20 h under an Ar atmosphere. After the reaction mixture was cooled to rt it was filtered through Celite and concentrated to dryness. The residue was purified by flash chromatography (33% ethyl acetate/hexane, then 50% ethyl acetate/hexane) to give **5** (4.69 g, 70%) as a colorless oil: ^{1}H NMR (CD_3OD, TMS): δ 1.28-1.80 (m, 6 H), 1.44 (s, 9 H), 1.45 (s, 9 H), 2.42-2.72 (m, 2 H),

3.22–3.47 (m, 4 H), 3.92 (m, 1 H, J = 8.4, 5.5), 4.08 (m, 1 H), 5.13 (s, 2 H), 7.20-7.38 (m, 5 H); ^{13}C NMR: δ 24.0, 24.2, 28.3, 28.7, 32.4, 38.4, 55.7, 67.1, 68.4, 80.4, 82.5, 118.9, 129.1, 129.2, 129.6, 137.9, 157.5–159.0 (br), 158.1, 173.8. $[\alpha]^{22}_{D}$ –18.1° (*c* 0.96, CH_3OH).

(2*S*,9*R*)-2-[(*tert*-Butoxycarbonyl)amino]-11-amino-9-hydroxy-7-azaundecanoic Acid *tert*-Butyl Ester, Diacetate Salt (6). A mixed catalyst of 10% Pd-C (0.50 g) and PtO_2 (0.93 g) was added to a solution of **5** (4.66 g, 8.97 mmol) in glacial acetic acid (112 mL). H_2 gas was introduced and after 24 h the catalysts were removed via filtration through Celite. The filtrate was concentrated in vacuo and excess acetic acid was removed via addition of toluene to form an azeotrope. This provided **6** as a colorless oil (4.57 g, 100%): ^{1}H NMR (D_2O, external TSP): δ 1.44 (s, 9 H), 1.47 (s, 9 H), 1.50-1.80 (m, 8 H), 2.04 (s, 6 H), 3.01-3.24 (m, 6 H), 3.97 (dd, 1 H, J = 9.1, 5.2), 4.06 (tt, 1 H, J = 9.6, 3.3); ^{13}C NMR (D_2O, internal standard CH_3OH = 49.5 ppm): δ 22.7, 24.2, 26.7, 28.3, 28.8, 29.3, 32.1, 33.2, 38.2, 53.9, 56.1, 66.4, 81.1, 84.5, 158.9, 175.0, 178.8; $[\alpha]^{25}_{D}$ –17.1° (*c* 1.00, CH_3OH).

(2*S*,9*R*)-11-[(Benzyloxycarbonyl)amino]-2-[(*tert*-butoxycarbonyl)amino]-9-hydroxy-7-(carbobenzyloxy)-7-azaundecanoic Acid *tert*-Butyl Ester (7). Compound **6** (4.57 g, 8.98 mmol) was dissolved in water (150 mL) and diethyl ether (150 mL). This biphasic solution was vigorously stirred and cooled to 0 °C under an Ar atmosphere. $KHCO_3$ (14.02 g, 140.0 mmol) was added to the solution. Then *N*-(benzyloxycarbonyloxy)-succinimide (CBZ-ONSu, 8.06 g, 32.33 mmol) was added in several portions over 20 min. The reaction mixture was allowed to warm to room temperature and stir for an additional 5 h. The layers were then separated and the

aqueous layer was extracted with diethyl ether (3 × 200 mL). Ether layers were combined, dried and concentrated. Purification of the residue by flash chromatography (50% ethyl acetate/hexane) gave **7** (3.83 mg, 65%) as a colorless oil: ^{1}H NMR (CD_3OD, TMS): δ 1.30-1.80 (m, 8 H), 1.43 (s, 9 H), 1.44 (s, 9 H), 3.11-3.44 (m, 6 H), 3.82 (m, 1 H), 3.92 (m, 1 H), 5.06 (s, 2 H), 5.10 (s, 2 H), 7.24 -7.38 (m, 10 H); ^{13}C NMR: δ 24.1, 28.3, 28.8, 32.5, 35.9, 38.6, 54.0, 54.7, 55.8, 67.4, 68.3, 69.0, 80.4, 82.5, 128.8, 128.9, 129.1, 129.5, 129.6, 138.1, 138.4, 158.1, 158.4, 158.9, 173.8; $[\alpha]^{22}_D$ –12.1° (*c* 1.00, CH_3OH).

Synthesis of α-(β-alanyl)- and α-(γ-aminobutyryl)-Hypusine

(2*S*,9*R*)-11-[(Benzyloxycarbonyl)amino]-2-amino-9-hydroxy-7-(carbobenzyloxy)-7-azaundecanoic Acid *tert*-Butyl Ester (12). The BOC protecting group was selectively removed from **7** (3.83 g, 5.82 mmol) via the addition of 5 mL of dry 1 M HCl in ethyl acetate (chilled) to six-638 mg fractions (approximately 1 mmol each) of the di-CBZ, Boc, *O*-*t*-Butyl protected hypusine reagent. The reaction mixtures were allowed to stir at room temperature under an Ar atmosphere and were monitored by TLC (9:1 $CHCl_3$/MeOH) until all starting material was consumed (i.e. converted to free amine). Typical reaction times were 3 to 4 h. The solutions were then concentrated to remove excess HCl/EtOAc and then dried under vacuum. The glass-like materials were then dissolved in ethyl acetate (45 mL), combined, and washed with saturated $NaHCO_3$ solution (3 x 50 mL). The aqueous and organic layers were separated followed by extraction of the aqueous layer with ethyl acetate (3 x 100 mL). The organic layers were combined and dried over Na_2SO_4, filtered, and concentrated to dryness. Purification by flash chromatography (90% MeOH:$CHCl_3$) yielded the free amine, a clear oil **12** (2.05g,

64%): ^{1}H NMR (CD_3OD, TMS): δ 1.28-1.76 (m, 8 H), 1.45 (s, 9 H), 3.11-3.45 (m, 7 H), 3.82 (m, 1 H), 5.06 (s, 2 H), 5.10 (s, 2 H), 7.29-7.34 (m, 10 H); ^{13}C NMR: δ 23.3, 23.7, 29.2, 35.5, 35.9, 38.6, 54.1, 54.8, 55.5, 67.4, 68.3, 68.9, 82.2, 128.8, 129.0, 129.1, 129.5, 129.6, 138.1, 138.4, 158.4, 158.9, 175.9; $[\alpha]^{22}_D$ +4.2° (*c* 1.00, CH_3OH). HRMS *m/z* calcd for $C_{30}H_{44}N_3O_7$ 558.3179, found 558.3181; Anal. ($C_{30}H_{43}N_3O_7$) C, H, N calcd: C 64.61, H 7.77, N 7.53, found C 63.59, H 7.67, N 7.26.

BOC-β-Alanyl-(2S,9R)-Hypusine-[N^7,N^{12}-di-CBZ]-9-hydroxy-O-*t*-Bu (13). A solution of **12** (249 mg, 0.45 mmol) and BOC-β-alanine-OH (110 mg, 0.58 mmol) in 5 mL of methylene chloride was cooled to 0 °C under an Ar atmosphere, and allowed to stir for 10 min. PyBOP® (348 mg, 0.67 mmol) coupling reagent was added followed by diisopropylethyl amine (101 mg, 0.78 mmol) 15 min later. The reaction mixture was allowed to stir for 10 min before warming to room temperature. After 1.5 hrs, the reaction mixture was diluted with saturated NaCl solution and extracted with $CHCl_3$ (3 x 20 mL). The combined organic layers were successively washed with ice-cold 10% citric acid/brine (1:1, 20 mL), brine (20 mL), saturated $NaHCO_3$ solution/brine solution (1:1, 20 mL), and brine (20 mL). The organic layer was dried with Na_2SO_4 and concentrated *in vacuo*. Purification via flash chromatography (17% hexane/ethyl acetate) yielded a colorless oil **13** (266 mg, 82%): ^{1}H NMR (CD_3OD, TMS): δ 1.30-1.80 (m, 8 H), 1.42 (s, 9H), 1.44 (s, 9 H), 2.40 (t, 2 H, J = 6.6) 3.11-3.48 (m, 8 H), 3.81 (s, br, 1 H), 4.22 (s, br, 1 H) 5.08 (s, 2 H), 5.11 (s, 2 H), 7.24-7.38 (m, 10 H); ^{13}C NMR: δ 24.1, 28.3, 28.8, 32.2, 35.9, 37.0, 38.0, 38.6, 54.1, 54.4, 54.6, 67.4, 68.3, 68.9, 80.2, 82.8, 128.8, 128.9, 129.1, 129.5, 129.6, 138.1, 138.4, 158.1, 158.4, 159.0, 173.1, 174.0; $[\alpha]^{19}_D$ -10.5° (*c* 1.00,

CH_3OH). HRMS *m/z* calcd for $C_{38}H_{57}N_4O_{10}$ 729.4075, found 729.4072; Anal. ($C_{38}H_{56}N_4O_{10}$) C, H, N calcd: C 62.62, H 7.74, N 7.69, found C 61.17, H 7.62, N 7.52.

BOC-γ-aminobutyryl-(2S,9R)-Hypusine-[N^7,N^{12}-di-CBZ]-9-hydroxy-O-*t*-Bu (14). According to the method described for the preparation of **13**, a solution of **12** (168 mg, 0.30 mmol) and BOC-γ-aminobutyric acid (80 mg, 0.58 mmol) in 5 mL of methylene chloride was reacted with PyBOP® (235 mg, 0.45 mmol) coupling reagent and diisopropyl ethyl amine (68 mg, 0.53 mmol) for 1.5 hr to obtain **14** as a colorless oil (173 mg, 77%): ^{1}H NMR (CD_3OD, TMS): δ 1.28-1.68 (m, 8 H), 1.74 (quintet, 2 H), 1.42 (s, 9H), 1.44 (s, 9 H), 2.23 (t, 2 H, J = 7.6) 3.06 (t, 2H, J = 6.9), 3.10-3.48 (m, 6 H), 3.74-3.88 (m, 1 H), 4.21 (t, br, 1 H, J =6.7) 5.06 (s, 2 H), 5.10 (s, 2 H), 7.24-7.40 (m, 10 H); ^{13}C NMR: δ 24.1, 27.4, 28.3, 28.8, 32.3, 34.0, 35.9, 38.6, 40.8, 54.1, 54.5, 54.7, 67.4, 68.3, 68.9, 80.0, 82.7, 128.8, 128.9, 129.1, 129.5, 129.6, 138.1, 138.4, 158.1, 158.6, 159.0, 173.1, 175.7; $[\alpha]^{19}_D$ -10.0° (*c* 1.00, CH_3OH). HRMS *m/z* calcd for $C_{39}H_{59}N_4O_{10}$ 743.4231, found 743.4211; Anal. ($C_{39}H_{58}N_4O_{10}$) C, H, N calcd: C 63.05, H 7.87, N 7.54, found C 63.27, H 7.98, N 7.39.

α-(β-alanyl)-(2S,9R)-hypusine trihydrochloride salt (15). Under an Ar atmosphere, phenol (2.40 g, 25 mmol), pentamethylbenzene (2.40 g, 16 mmol), and **13** (250 mg, 0.34 mmol) were dissolved in TFA (30 mL), cooled to 0 °C and allowed to stir for 20 min. 30% HBr/HOAc (1.08 mL) was added and the solution was allowed to stir for an additional 10 min before being warmed to room temperature. After 1.25 h, the reaction mixture was concentrated, diluted with 10% HOAc/H_2O (45 mL), and extracted with methyl *tert*-butyl ether (3 x 45 mL). The aqueous layer was then concentrated and purified via ion exchange chromatography using consecutive column washes with 0.1 N

to 6 N HCl (aq) to elute the peptide. The compound eluted with 6 N HCl (150mL). Fractions containing the product (TLC/ninhydrin detection) were combined and concentrated to yield **15** (130 mg, 92%) as a hygroscopic, glass-like salt: ^{1}H NMR (D_2O, NaTSP external reference): δ 1.43-1.53 (m, 2 H), 1.70-2.0 (m, 6 H), 2.75 (t, 2 H, J = 6.7) 3.01-3.23 (m, 8 H), 4.06 (tt, 1 H, J =9.6, 3.2), 4.38 (dd, 1 H, J =8.7, 5.4); ^{13}C NMR (D_2O, NaTSP external reference): δ 23.1, 25.7, 30.8, 32.3, 32.5, 36.5, 37.4, 48.3, 52.9, 53.5, 65.6, 173.2, 176.6; $[\alpha]^{24}_{D}$ -9.1° (*c* 1.00, 6N HCl). TLC (2:2:1 CH_2Cl_2/MeOH/NH_4OH, R_f = 0.20, violet spot; Citric Acid (2.46g)/HCl (0.65mL)/NaOH (1.40g)/H_2O (qs to 100 mL), Rf = 0.30, violet spot). HRMS *m/z* calcd for $C_{13}H_{29}N_4O_{10}$ 305.2189, found 305.2165; Anal. ($C_{13}H_{28}N_4O_{10}\cdot$ 3HCl) C, H, N, Cl calcd: C 37.74, H 7.55, N 13.54, Cl 25.70 found C 35.44, H 7.73, N 12.36, Cl 28.86.

α-(γ-aminobutyryl)-(2S,9R)-hypusine trihydrochloride salt (16). Under an Ar atmosphere, phenol (1.5 g, 16 mmol), pentamethylbenzene (1.5 g, 10 mmol), and **14** (169 mg, 0.23 mmol) were dissolved in TFA (21 mL), cooled to 0 °C and reacted with 30% HBr/HOAc (0.73 mL) as described for **15**. Purification via ion exchange chromatography yielded **16** (91.3mg, 93%), as a glass-like salt: ^{1}H NMR (D_2O, NaTSP external reference): δ 1.43-1.53 (m, 2 H), 1.70-2.01 (m, 8 H), 2.45 (t, 2 H, J = 7.4) 3.00-3.23 (m, 8H), 4.06 (tt, 1 H, J =9.6, 3.2), 4.35 (dd, 1 H, J =8.8, 5.3); ^{13}C NMR: δ 23.1, 23.7, 25.7, 30.7, 32.2, 32.9, 37.3, 39.7, 48.3, 52.9, 53.5, 65.6, 176.0, 176.6; $[\alpha]^{24}_{D}$ -10.9° (*c* 1.00, 6N HCl). TLC (2:2:1 CH_2Cl_2/MeOH/NH_4OH, R_f = 0.19, red spot; Citric Acid (2.46g)/HCl (0.65mL)/NaOH (1.40g)/H_2O (qs to 100 mL), R_f = 0.28, pink spot): HRMS *m/z* calcd for $C_{14}H_{31}N_4O_{10}$ 319.2345, found 319.2348; Anal. ($C_{13}H_{30}N_4O_4\cdot$ 3HCl) C, H,

N, Cl calcd: C 39.31, H 7.78, N 13.10, Cl 24.86 found C 34.62, H 7.67, N 11.43, Cl 29.04.

Synthesis of ^{3}H-Hypusine

(2S, 9R)-2,11-diamino-9-hydroxy-7-azaundecanoic acid (^{3}H at C-11) (18). A portion of **5** along with Pd-C and PtO_2 was dissolved in acetic acid and reduced with $^3H_2$ at Moravek Biochemicals (Brea, CA). The product of this reduction was purified as described for **6** above. The radiochemical purity of **6b** was determined to be 98% by TLC in 4:4:1 ethanol/dichloromethane/ammonium hydroxide. Approximately 1.0 mCi/ml (649.2 μg/mL) solution of ^{3}H-labeled hypusine precursor (specific activity: 600 mCi/mmol) was now available for use in the preparation of ^{3}H-hypusine.

Due to the high radioactivity achieved by the $^3H_2$ reduction of compound **5** to achieve **6b**, a "cold dilution" step was incorporated before the final conversion to radiolabeled hypusine (**18**). Unlabeled compound **6** (50 mg, 0.128 mmol) was added to 1.5 mL of the radiolabeled **6b** solution in ethanol. This dilution process resulted in a specific activity of 16 mCi/mmol (almost a 38 fold dilution from the stock **6b**). Total DPM of the starting solution was 4.67×10^9 DPM. The solution was concentrated to dryness followed by azeotropic removal of excess ethanol with CH_2Cl_2. Methylene chloride (5 mL) was added and cooled. TFA (30 μL) was added and the reaction was stirred under N_2 at rt for approximately one hr. The solution was again concentrated to dryness followed by the addition of TFA (5 mL). The solution was allowed to stir at rt under N_2 for approximately three hr. In order to determine when all starting material had been fully deprotected, the reaction was monitored by TLC (2:1:1 $MeOH/CH_2Cl_2/NH_4OH$). Again the contents of the reaction flask were concentrated to

dryness followed by 3 x 10 mL methylene chloride washes. The residue was purified by small-scale flash chromatography (15 x 2.5 cm, flash silica, 2:1:1 methanol/methylene chloride/ammonium hydroxide). As the radiolabeled compound eluted from the column, the fractions collected were analyzed by TLC and scintillation counting as described in Chapter 2 (Fig. 2-5). A colorless oil, **18** was then acidified with 0.1 N HCl (aq) and transferred to vials for storage at 0 °C. Total DPM obtained = 1.75×10^9, 37.5 % yield.

Solid Phase Peptide Synthesis–Use of Hypusine and Deoxyhypusine Reagents

The Fmoc-Leu-Wang Resin (1.0g) was placed in the reaction vessel and solvated or "swelled" for three hours in DMF (dry, distilled) with N_2 bubbling. Afterwards, the resin was washed five times with excess DMF. The terminal Fmoc was then removed via the addition of approximately 20 mL 20-30% piperidine in DMF first for two minutes then 25 minutes. The resin was then rinsed four times with excess DMF. A small portion of the resin was removed from the reaction batch via pipette tip and transferred to a glass test tube where the presence of a free N^{α} amine was confirmed by the Kaiser (ninhydrin) test.

It should be noted that occasionally during SPPS, couplings did not go to completion after a 2-3 hr coupling time with 5-fold excess reagents. Recouplings (the drainage followed by the readdition of coupling reagents) were employed to drive reactions to completion. To aid in this matter, larger excesses of reagents (sometimes up to 20-fold) and longer reaction times (sometimes up to 72 hours total, with refreshing of reagents at least every 24 hours) were sometimes used. Also, it is well known that as peptide chains elongate on the solid phase resin, they may aggregate. Therefore additional reagents such as 20% trifluoroethanol and 0.4M LiCl were used as needed to

help break up the formation of any aggregation (clumped resin), though not mentioned specifically below. Alternative coupling reagents and solvents like BOP, PyBOP®, and CH_2Cl_2 (as opposed to DMF) were also utilized when necessary. The eIF-5A peptide fragments were analyzed on the 500 MHz NMR (^1H, 5 mm TXI probe; ^{13}C, 5 mm BBO probe) at the UF McKnight Brain Institute AMRIS facility unless otherwise noted below.

NH_2-Ala$_{54}$-Lys$_{55}$-Val$_{56}$-His$_{57}$-Leu$_{58}$-OH (20). A five-fold excess of Fmoc-His$_{57}$(Trt) (1.115 g, 1.80 mmol) and HBTU (0.683 g , 1.80 mmol) were dissolved completely in 3 mL of DMF. After two minutes, 440 μL of DIEA (2.5 mmol, d=0.741) were added to the solution. The Fmoc-His(Trt)/HBTU/DIEA solution was then transferred to the deprotected NH_2-Leu$_{58}$-Wang Resin. An additional 5 mL of DMF were added to the resin in the reaction vessel. N_2 was introduced to facilitate mixing. After approximately two hours the DMF solution was drained and the resin was washed three times with excess DMF. A small portion of the resin was removed from the reaction batch via pipette tip and transferred to a glass test tube where the absence of a free N^{α} amine, or complete coupling, was confirmed by the Kaiser (ninhydrin) test (colorless beads, golden yellow solution). After the reaction was deemed complete by the Kaiser test, 20 mL of a 2:1:2 capping solution of acetic anhydride/diisopropylethylamine/methylene chloride was added to the resin for approximately 30 min. The solution was drained and the resin was washed three times with excess CH_2Cl_2, two times with excess MeOH, and three times with excess CH_2Cl_2. The resin was then washed five times with excess DMF prior to the next deprotection. This cycle of deprotection/coupling/capping was repeated as described above using the following protected amino acids (all in 5-fold excess) in the sequence (side chain

protecting groups are in parentheses): Fmoc-Val_{56} (0.611 g, 1.8 mmol), Fmoc-Lys_{55}(Boc) (0.843 g, 1.80 mmol), Fmoc-Ala_{54} (0.593 g , 1.80 mmol).

Once the final coupling cycle was completed, the resin was washed with excess CH_2Cl_2 and allowed to dry in a desiccator under vacuum overnight. Then, 120 mg of the Fmoc-Ala_{54}—Leu_{58}-Wang Resin (0.0337 mmol Fmoc-peptide) were removed from the batch and placed in a separate reaction vessel. The terminal Fmoc protecting group was removed in the same manner as above and verified by the Kaiser test. The resin was then washed with excess DMF, CH_2Cl_2, MeOH, and CH_2Cl_2 three times each. A cleavage cocktail (95% trifluoroacetic acid, 2.5% H_2O, and 2.5% triisopropylsilane) was added to the resin and mixed with a gentle N_2 gas flow. After 2.5 hr, the solution was drained and collected into a tared 50 mL centrifuge tube. The resin was washed three times with excess cleavage cocktail. All washes were pooled and then partitioned into 3-4 centrifuge tubes. Cold ether (40 mL) was added to each tube in order to precipitate the peptide and extract scavengers and side chain protecting groups. The tubes were sealed, shaken, and then centrifuged for 15 min at 2500 rpm, 10 °C. The ether was carefully decanted leaving a white residue in the centrifuge tubes. A total of three cold ether extractions were carried out. The peptide residues were allowed to dry *in vacuo* overnight.

The crude peptide residue was then purified via preparative HPLC with UV detection at 220 nm as discussed in the Analytical chapter. Collected fractions were then analyzed by analytical scale HPLC at 220 nm to determine which fractions contained the desired peptide. The fractions containing the main peptide peak were then pooled and lyophilized to give **20** as a white powder (15.5 mg, 0.0274 mmol, 81.0% yield). Mass

expected: 566.69, observed: 568.88 (m/z = +1), 608.08 (m+K, potassium ion). Purity: > 98% by HPLC. ^{1}H NMR (7.7.mM; 3.5 mg in 0.80 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.90; 7 °C): δ 0.8567-0.9561 (dd, 12 H, J = 21.52/5.91, 15.46/6.85), 1.3972-1.8710 (m, 9 H), 1.5206-1.5348 (d, 3 H, J = 7.1), 1.9952-2.0368 (m, 1 H), 3.0007 (br s, 2 H), 3.1773-3.2732 (dd, 2 H, J=15.40/6.80, 15.35/7.75), 4.0649-4.1071 (m, 2 H), 4.2698-4.3132 (m, 1 H), 4.3585-4.4001 (m, 1 H), 4.6889-4.7326 (m, 1 H), 7.3266 (s, 1 H), 7.6201 (br s), 8.4162-8.4305 (d, 1 H, J = 7.1), 8.5759-8.5908 (d, 1 H, J = 7.4), 8.6194 (s, 1 H), 8.7215-8.7344 (d, 1 H, J = 6.4), 8.7549-8.7701 (d, 1 H, J = 7.6); ^{13}C NMR (7.7.mM; 3.5 mg in 0.80 mL 90% H_2O/ 10% D_2O; external NaTSP, pH = 2.90; 7 °C): δ 19.707, 20.865, 21.197, 23.332, 25.087, 25.309, 27.357, 29.410, 29.460, 33.246, 33.444, 42.337, 42.556, 51.938, 55.346, 55.559, 56.591, 62.551, 115.760**, 118.077**, 120.394**, 120.534, 122.711**, 131.004, 136.682, 165.639*, 165.922*, 166.206*, 166.490*, 173.852, 173.979, 175.878, 176.736, 180.834 (peaks marked with * and ** indicate TFA COOH, J = 106.9/71.3/35.7 and TFA CF_3, J = 583.1/291.5 respectively). Additional 2D NMR data is reported in Chapter 6.

NH_2-His_{51}-Gly_{52}-His_{53}-Ala_{54}-Lys_{55}-Val_{56}-His_{57}-Leu_{58}-OH (21). The remainder of the resin used for the synthesis of **20** (1.160 g resin/peptide, 0.3689 g peptide, 0.3261 mmol) was used for the synthesis of **21**. The peptide sequence was elongated through a series of deprotection/coupling/capping cycles as for **20** using the following protected amino acids in the sequence: Fmoc-His_{53}(Trt) (1.12 g, 1.80 mmol), Fmoc-Gly_{52} (0.535 g, 1.80 mmol), Fmoc- His_{51}(Trt) (1.12 g, 1.80 mmol). Once the final coupling cycle was completed, the resin was washed with excess CH_2Cl_2 and allowed to dry under vacuum overnight. Then, 120 mg (0.0274 mmol Fmoc-peptide) of the Fmoc-His_{51}(Trt)—Leu_{58}-

Wang Resin were removed from the batch and placed in a separate reaction vessel. The terminal Fmoc group was removed in the typical manner followed by the simultaneous side-chain deprotection and cleavage of the peptide from the resin via addition of the TFA/H_2O/TIS 95:2.5:2.5 cleavage cocktail for 2.5 hr. Cold ether extraction, purification by HPLC, and lyophilization yielded **21** as a white powder (23.9 mg, 0.0266 mmol, 97.0% yield). Mass expected: 898.02, observed: 897.30 (m/z = +1). Purity: > 97%, by HPLC. ^{1}H NMR (7.5 mM; 5.4 mg in 0.80 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.80; 7 °C): δ 0.8479-0.9363 (m, 12 H), 1.3781-1.3924 (d, 3 H, J = 7.15), 1.3409-1.8367 (m, 9 H), 1.9884-2.0286 (m, 1 H), 3.0107 (br s, 2 H), 3.1381-3.2934 (m, 4 H), 3.4095-3.4222 (d, 2 H, J = 6.35), 3.9990-4.0214 (t, 2 H, J = 11.21, 5.61), 4.0641-4.0940 (t, 1 H, J = 14.81, 7.52), 4.2724-4.3131 (m, 3 H), 4.3561-4.3817 (t, 1 H, J = 12.82, 6.41), 7.3170 (s, 1 H), 7.3276 (s, 1 H), 7.4535 (s, 1 H), 7.6181 (br s), 8.3478-8.3623 (d, 1 H, J = 7.25), 8.5875-8.6505 (m, 5 H), 8.6996 (s, 1 H), 8.7465-8.7677 (m, 2 H), 8.9203-8.9431 (t, 1 H, J = 11.42, 5.71); ^{13}C NMR (7.5 mM; 5.4 mg in 0.80 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.80; 7 °C): δ 19.590, 21.233, 23.324, 25.167, 25.297, 27.340, 29.133, 29.452, 29.461, 29.589, 33.323, 42.364, 42.504, 45.040, 52.709, 55.051, 55.279, 55.374, 55.421, 56.630, 62.457, 115.759**, 118.075**, 120.392**, 120.467, 120.523, 121.742, 122.709**, 128.643, 131.010, 131.265, 136.675, 137.661, 165.622*, 165.905*, 166.188*, 166.471*, 171.901, 173.732, 174.005, 174.427, 175.900, 176.879, 177.995, 180.628 (peaks marked with * and ** indicate TFA COOH, J = 106.9/71.3/35.7 and TFA CF_3, J = 583.1/291.5 respectively). Additional 2D NMR data is reported in Chapter 6.

NH_2-Thr_{45}-Ser_{46}-Lys_{47}-Thr_{48}-Gly_{49}-Lys_{50}-His_{51}-Gly_{52}-His_{53}-Ala_{54}-Lys_{55}-Val_{56}-His_{57}-Leu_{58}-OH (22). The following protected amino acids in the eIF-5A sequence

were sequentially coupled to 0.2984 g (0.1328 g peptide, 0.0682 mmol) of the Fmoc-His_{51}(Trt)—Leu_{58}-Wang Resin with HBTU and DIEA as for **20** and **21**: Fmoc-Lys_{50}(Boc) (0.162 g, 0.341 mmol), Fmoc-Gly_{49} (0.103g, 0.341 mmol), Fmoc-Thr_{48}(*t*-Bu) (0.138g, 0.341 mmol), Fmoc-Lys_{47}(Boc) (0.162g, 0.341 mmol), Fmoc-Ser_{46}(*t*-Bu) (0.133g, 0.341 mmol), Fmoc-Thr_{45}(*t*-Bu) (0.138, 0.341 mmol). It should be noted that longer coupling times and larger excesses of reagents were used to drive the couplings of Ser_{46} and Thr_{45} to completion (53 and 50 total hrs respectively, from 5- to 20-fold excess reagents). Capping procedures were followed after each coupling. Upon completion of the final coupling for this peptide, the resin was washed and dried. Approximately 100 mg of the Fmoc-Thr_{45}(*t*-Bu)—Leu_{58}-Wang Resin were removed from the batch and placed in a separate reaction vessel. The terminal Fmoc group was removed as above and the peptide was deprotected and cleaved from the resin using the TFA cleavage cocktail. Cold ether extraction, purification by HPLC, and lyophilization yielded **22** as a white powder (25.5 mg, 0.017 mmol, 90.9% yield). Mass expected: 1500.71, observed: 1499.8851 (m/z = +1), 1534.9640 (m+Cl, chloride ion). Purity: > 99%, by HPLC. ^{1}H NMR (5.9 mM; 4.4 mg in 0.5 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.85; 7 °C): δ 0.849-0.938 (m 12 H), 1.211-1.224 (d, 3 H, J = 6.3), 1.332-1.344 (d, 3 H, J = 6.4), 1.381-1.395 (d, 3 H, J = 7.2), 1.310-1.852 (m, 20 H), 1.860-2.059 (m, 2 H), 3.001 (br s, 6 H), 3.125-3.365 (m, 6 H), 3.852-4.050 (m, 6 H), 4.066-4.095 (t, 1 H, J = 14.8, 7.3), 4.163-4.485 (m, 9 H), 4.512-4.598 (m, 1 H), 4.654-4.794 (m, 3 H), 7.302 (s, 1 H), 7.318 (s, 2 H), 7.608 (br s), 8.272-8.287 (d, J = 7.4), 8.352-8.392 (m, 2 H), 8.450-8.580 (m, 2 H), 8.6196 (br s, 7 H), 8.749-8.808 (m, 4 H), 9.004-9.017 (d, 1 H, J = 6.4); ^{13}C NMR (5.9 mM; 4.4 mg in 0.5 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 7.05; 7

°C): δ 19.484, 20.740, 21.284, 21.512, 21.730, 23.640, 24.863, 25.054, 25.132, 25.451, 27.461, 29.308, 29.386, 33.206, 33.320, 42.337, 43.484, 45.425, 52.701, 56.122, 56.604, 56.830, 57.054, 58.565, 62.088, 62.353, 62.490, 64.084, 70.141, 70.936, 115.760**, 118.077**, 120.208, 120.394**, 122.711**, 138.382, 138.868, 165.642*, 165.927*, 166.210*, 166.493*, 174.136, 174.431, 175.381, 175.826, 176.400, 176.690, 176.818, 177.183, 177.755, 181.967, 182.775, 185.787 (peaks marked with * and ** indicate TFA COOH, J = 107.0/71.2/35.7 and TFA CF_3, J = 583.1/291.5 respectively). Additional 2D NMR data is reported in Chapter 6. Confirmed Amino Acid Sequence: NH_2-T-S-K-T-G-K-H-G-H-A-K-V-H-L-OH.

NH_2-Lys_{39}-Ile_{40}-Val_{41}-Glu_{42}-Met_{43}-Ser_{44}-Thr_{45}-Ser_{46}-Lys_{47}-Thr_{48}-Gly_{49}-Lys_{50}-His_{51}-Gly_{52}-His_{53}-Ala_{54}-Lys_{55}-Val_{56}-His_{57}-Leu_{58}-OH (23). The eIF-5A 20 mer containing Lys_{50} was prepared using standard Fmoc SPPS chemistry stemming from 0.2910 g (0.1295 g peptide, 0.0665 mmol) of the Fmoc-His_{51}(Trt)—Leu_{58}-Wang Resin (see **21**). BOP (0.147 g, 0.332 mmol), 1 M HOBt (1M, 340 µL), and DIEA (100 µL) were used as coupling reagents. The amino acids Fmoc-Lys_{50}(Boc) through Fmoc-Thr_{45}(*t*-Bu) were coupled to the resin as for **22**. Excess reagents and reaction times were again required for residues 46 and 45 as for **22**. Longer coupling times (3-4 hrs) were therefore allowed for the remaining amino acids (Fmoc-Ser_{44}(*t*-Bu) through Fmoc-Lys_{39}(Boc)), which proceeded without any hindrance. The terminal Fmoc group was removed as above and the peptide was deprotected and cleaved from the resin using the TFA cleavage cocktail. The crude peptide residue was extracted with cold ether and purified via HPLC as above to give **23** (11.0 mg, 0.0050 mmol, 7.6% yield) as a white powder. Mass expected: 2188.55, observed: 2188.55 (m/z = +1), 2209.99 (m+Na,

sodium ion), 1094.46 (m/z=+2). Purity: ~95%, by HPLC. ^{1}H NMR (4.9 mM; 8.8 mg in 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.82; 7 °C): δ 0.8623-0.9557 (m, 12 H), 1.2202 (strong singlet, *t*-butyl impurity), 1.381-1.3445 (m, beneath singlet, 8 H), 1.3842-1.3983 (d, 3 H, J = 7.0), 1.3445-1.8132 (m, 26 H), 1.8844-2.0983 (m, 8 H), 2.0983 (sh s, 3 H), 2.4463-2.6516 (m, 4 H), 2.9250-3.0890 (br s, 8 H), 3.0079-3.3585 (m, 6 H), 3.7933-4.0034 (m, 8 H), 4.0034-4.5441 (m, 17 H), 4.7054-4.7437 (m, 3 H), 7.3065 (s, 1 H), 7.3205(s, 2 H), 7.6195 (br s), 8.1896-8.2041 (d, J = 7.2), 8.6228 (br s, 3 H), 8.3423-8.7777 (m, amide protons); ^{13}C NMR (4.9 mM; 8.8 mg in 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.82; 7 °C): δ12.966, 17.133, 17.565, 19.586, 20.823, 21.011, 21.239, 21.335, 21.780, 21.788, 23.327, 24.152, 25.031, 25.154, 25.298, 27.337, 27.600, 29.299, 32.145, 32.881, 33.133, 33.316, 33.587, 36.068, 39.009, 42.200, 42.364, 42.483, 45.375, 45.396, 52.742, 55.124, 55.242, 55.365, 55.609, 55.634, 55.822, 56.595, 56.606, 57.824, 58.196, 58.603, 61.379, 62.026, 62.222, 62.448, 63.002, 63.975, 70.012, 70.076, 77.882, 115.768**, 118.085**, 120.403**, 120.526, 120.625/620, 122.718**, 131.029, 131.350, 131.438, 136.669, 165.631*, 165.914*, 166.197*, 166.480*, 172.646, 173.916, 174.026, 174.335, 174.449, 174.898, 175.087/119, 175.272/273, 175.432, 175.432, 175.896, 176.013, 176.125, 176.581, 176.863, 176.990, 177.983, 180.169, 180.549 (peaks marked with * and ** indicate TFA COOH, J = 106.7/71.1/35.6 and TFA CF_3, J = 583.2/291.6 respectively). Additional 2D NMR data is reported in Chapter 6. Confirmed Amino Acid sequence: NH_2-K-I-V-E-M-S-T-S-K-T-G-K-H-G-H-A-K-V-X-X-OH (X = not detected–sequencing was able to analyze the peptide out to Val_{56}. The sample washed off column afterwards.).

NH_2-Lys_{39}-Ile_{40}-Val_{41}-Glu_{42}-Met_{43}-Ser_{44}-Thr_{45}-Ser_{46}-Lys_{47}-Thr_{48}-Gly_{49}-DeoxyHypusine$_{50}$-His_{51}-Gly_{52}-His_{53}-Ala_{54}-Lys_{55}-Val_{56}-His_{57}-Leu_{58}-OH (24). The eIF-5A 20mer containing Deoxyhypusine$_{50}$ was prepared using standard Fmoc SPPS chemistry procedures. Fmoc-Deoxyhypusine$_{50}$ (**19**, 0.114g, 0.144 mmol), HBTU (0.055 g, 0.144 mmol), and 30 µl of DIEA were reacted with 0.300 g (0.1335 g peptide, 0.0686 mmol) of the Fmoc-His_{51}(Trt)—Leu_{58}-Wang Resin (see **21**) for 2 hr. The remaining amino acids in the sequence were coupled as above (with 5-20 fold excess of reagents as needed). Several residues required much longer coupling times similar to those experienced during the synthesis of **23**: Ser_{46}, 21 hrs; Thr_{45}, 20 hrs; Ser_{44}, 24 hrs ; Met43, 3.5 hrs; Glu_{42}, 17 hrs; Ile_{40}, 17 hrs; Lys_{39}, 4 hrs. The resin was subjected to the capping procedure described for **21** after each coupling reaction was completed. After successful completion of the final coupling, the terminal Fmoc protecting group was removed from approximately 0.445 g of resin/peptide (0.278 g peptide, 0.0686 mmol). Phenol and pentamethylbenzene (400 mg each) were added to the resin in a flask and cooled in an ice bath. A cleavage cocktail consisting of 10 mL TFA and 500 µL of TIS were then added and the mixture was stirred for 5 min under N_2 atmosphere. Then, 400 µL of 30% HBr in acetic acid were added. After 15 minutes the reaction was allowed to warm to rt and stir under N_2 for 1.5 hours. The resin beads were filtered from the flask and the solution was concentrated to near dryness via rotovap. Approximately 20 mL of 10% acetic acid was added to the residue. The solution was then extracted with 3 x 30 mL diethylether. The aqueous layer was then lyophilized to yield an orange/brown residue. The crude peptide was then purified via HPLC as previously described to yield **24** as a white powder (28.6 mg, 0.0127 mmol, 18.4% yield). Mass expected: 2259.68, observed: 2258.85 (m/z =

+1), 2281.80 (m + Na, sodium ion), 1130.02 (m/z = +2), 567.98 (m/z = +4). Purity: >92%, by HPLC. ^{1}H NMR (4.0 mM; 6.8 mg in 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.83; 7 °C): δ 0.8588-0.9560 (m, 24 H), 1.2122-1.2344 (t, 6 H, J = 11.1, 5.6), 1.2714-1.5500 (m, 10 H), 1.3833-1.3974 (d, 3 H, J = 7.0), 1.5520-1.9302 (m, 20 H), 1.9302-2.1490, (m, 8 H), 2.0996 (s, 3 H), 2.4410-2.6370 (m, 4 H), 2.9525-3.1420 (br s, 6 H), 2.9844 (sh s, 6 H), 3.1420-3.3595 (m, 6 H), 3.853-3.953 (m, 8 H), 3.953-4.625 (m, 17 H), 4.700-4.758 (m, 3 H), 7.304 (s, 2 H), 7.322 (s, 1 H), 7.625 (br s, 7.55-7.80), 8.182-8.1.97 (d, J = 7.3), 8.620 (br s, 3 H), 8.310-8.850 (m); ^{13}C NMR (4.0 mM; 6.8 mg in 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.83; 7 °C): δ 12.971, 17.125, 17.567, 19.628, 20.819, 21.011, 21.249, 21.333, 21.789, 21.793, 23.345, 24.165, 25.163, 25.309, 25.867, 27.061, 27.351, 27.606, 28.066, 29.301, 29.470, 32.146, 32.942, 33.136, 33.330, 33.593, 39.014, 40.115, 41.992, 42.200, 42.369, 42.525, 45.225, 45.387, 50.012, 50.475, 52.714, 55.234, 55.376, 55.486, 55.678, 55.797, 56.636, 58.602, 58.849, 61.377, 62.064, 62.205, 62.453, 63.986, 70.075, 70.098, 115.767**, 118.087**, 120.405**, 120.451, 120.538, 122.722**, 131.016, 131.326, 131.405, 136.392, 165.615*, 165.898*, 166.182*, 166.464*, 172.650, 173.873, 173.998, 174.311, 174.426, 175.097, 175.296, 175.428, 175.888, 175.888, 176.013, 176.126, 176.866, 176.924, 177.327, 177.961, 180.236, 180.706 (peaks marked with * and ** indicate TFA COOH, J = 106.9/71.3/35.6 and TFA CF_3, J = 583.1/291.7 respectively). Additional 2D NMR data is reported in Chapter 6. Confirmed Amino Acid Sequence: NH_2-K-I-V-G-M-S-T-S-K-T-G-$X_{(50)}$-H-G-H-A-K-V-H-L-OH (No amino acid was reported at position 50 as expected since the automated sequencer does not have a deoxyhypusine internal standard).

NH_2-Lys_{39}-Ile_{40}-Val_{41}-Glu_{42}-Met_{43}-Ser_{44}-Thr_{45}-Ser_{46}-Lys_{47}-Thr_{48}-Gly_{49}-Hypusine$_{50}$-His_{51}-Gly_{52}-His_{53}-Ala_{54}-Lys_{55}-Val_{56}-His_{57}-Leu_{58}-OH (25). The eIF-5A 20 mer containing Hypusine$_{50}$ was synthesized beginning with 0.3038 g (0.1360 g peptide, 0.069 mmol) of Fmoc-His_{51}—Leu_{58}-Resin (see **21**). Fmoc-Hypusine$_{50}$ (**10**, 0.220 g, 0.272 mmol) was substituted for Fmoc-Lys_{50}(Boc). HBTU (0.1312 g, 0.346 mmol) was used as the coupling reagent along with 85 μL DIEA. The remaining amino acids in the sequence were coupled as above (with 5-20 fold excess of reagents). Coupling times were typically 2—2.5 hrs for Hypusine$_{50}$ through Lysine$_{47}$, Met_{43}, and Val_{41}. Several residues required much longer coupling times similar to those experienced during the synthesis of **23**: Ser_{46}, 22 hrs; Thr_{45}, 5.5 hrs; Ser_{44}, 17.5 hrs; Glu_{42}, 19 hrs; Ile_{40}, 20 hrs; Lys_{39}, 4 hrs. The resin was capped after each coupling reaction was completed.

After successful completion of the final coupling, the terminal Fmoc protecting group was removed from approximately 0.160 g of resin/peptide (55.3 mg peptide, 0.0243 mmol). The entire peptide was deprotected and cleaved from the resin using the HBr/HOAc/TFA/phenol/pentamethylbenzene cocktail and purified by ether extraction and HPLC as for **24** to give a white powder **25** (11.5 mg, 0.0051 mmol, 20.8% yield). Mass expected: 2275.68, observed: 2276.71 (m/z = +1), 2261.63 (m-OH), 1139.82 (m/z=+2). Purity: >90%, by HPLC. ^{1}H NMR (5.0 mM; 6.8 mg in 0.6 mL 90% H_2O/ 10%D_2O; external NaTSP; pH = 2.62; 7 °C): δ 0.8542-0.9593 (m, 24 H), 1.1669-1.3856 (m, 5 H), 1.3856-1.5066 (m, 9 H), 1.3856-1.3997 (d, 3 H, J = 7.1), 1.5066-1.8620 (m, 21 H), 1.8620-2.1181 (m, 11 H), 2.1099 (s, 3 H), 2.4340-2.666 (m, 4 H), 3.0111 (br s, 10 H), 3.1453-3.3630 (m, 8 H), 3.8425-4.1162 (m, 8 H), 4.1162-4.6000 (m, 18 H), 4.7076-4.7461 (m, 3 H), 7.308 (s, 2 H), 7.323 (s, 1 H), 7.631 (br s), 7.731 (br s), 8.627 (br s, 3

H), 8.186-8.797 (m); for ^{13}C NMR data for the eIF-5A 20mer with $Hypusine_{50}$, see **26**. Additional 2D NMR data as well as pH titration NMR data is reported in Chapter 6-NMR. Confirmed Amino Acid Sequence: NH_2-K-I-V-G-M-S-T-S-K-T-G-$X_{(50)}$-H-G-H-A-X-X-X-X-OH (Sequencing was able to report peptide sequence up to Ala_{54}. Sample washed off column afterwards. No amino acid was reported at position 50 as expected since the automated sequencer does not have hypusine as an internal standard). Amino Acid Analysis (expected, observed): Glu: (1, 0.7107); Ser: (2, 1.878); Gly: (2, 1.9915); His: (3, 3.1156); Thr: (2, 1.9054); Ala: (1, 1.1636); Val: (2, 2.1124); Met: (1, 0.3372); Ile: (1, 1.0295); Leu: (1, 1.2058); Lys: (3, 2.9649).

NH_2-Lys_{39}-Ile_{40}-Val_{41}-Glu_{42}-Met_{43}-Ser_{44}-Thr_{45}-Ser_{46}-Lys_{47}-Thr_{48}-Gly_{49}-(^{13}C)$Hypusine_{50}$-His_{51}-Gly_{52}-His_{53}-Ala_{54}-Lys_{55}-Val_{56}-His_{57}-Leu_{58}-OH (26). The eIF-5A 20 mer containing ^{13}C-Hypusine at position 50 was synthesized beginning with 0.270 g (0.1202 g peptide, 0.062 mmol) of Fmoc-His_{51}—Leu_{58}-Resin (see **21**). Fmoc-^{13}C-$Hypusine_{50}$ (**10a**, 0.075g, 0.092 mmol) was substituted for Fmoc-Lys_{50}(Boc). HBTU (0.035 g, 0.346 mmol) was used as the coupling reagent along with 25 μL DIEA. The remaining amino acids in the sequence were coupled as above (with 5-20 fold excess of reagents). Coupling times were typically 2-3 hrs for ^{13}C-$Hypusine_{50}$ through $Lysine_{47}$, Met_{43}, and Val_{41}. Residues Ser_{46} and Thr_{45} were coupled for 72 hrs each. Residues Ser_{44}, Met_{43}, Glu_{42}, and Ile_{40} were reacted overnight. Val_{41} and Lys_{39} ran for 4 hrs each. The resin was subjected to the capping procedure after each coupling reaction was completed.

After successful completion of the final coupling, the terminal Fmoc protecting group was removed from 0.160 g of resin/peptide (0.0553 g peptide, 0.0243 mmol). The entire peptide was deprotected and cleaved from the resin and purified as for **24** to give a

white powder **25** (11.5 mg, 0.0051 mmol, 20.8% yield). Mass expected: 2275.68, observed: 2276.71 (m/z = +1), 2261.63 (m-OH), 1139.82 (m/z = +2). Purity: >90%, by HPLC. ^{1}H NMR (6.3 mM; 7.2 mg in 0.5 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.95; 7 °C): δ 0.8563-0.9544 (m, 24 H), (1.15) 1.2095-1.2326 (t over m, 5 H, J = 11.56, 5.78), 1.3856-1.5066 (m, 9 H), 1.3810-1.3951 (d, 3 H, J = 7.1), 1.5066-1.8620 (m, 21 H), 1.8620-2.056 (m, 11 H), 2.0971 (s, 3 H), 2.4340-2.666 (m, 4 H), 3.0064 (br s, 10 H), 3.1402-3.3572 (m, 8 H), 3.8325-4.150 (m, 8 H), 4.150-4.6000 (m, 18 H), 4.675-4.775 (m, 3 H), 7.3016 (s, 2 H), 7.3191 (s, 1 H), 7.628 (br s), 7.727 (br s), 8.6243 (br s, 3 H), 8.1795-8.797 (m). ^{13}C NMR (6.3 mM; 7.2 mg in 0.5 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 2.95; 7 °C): δ12.969, 17.137, 17.566, 19.622, 20.813, 21.012, 21.248, 21.342, 21.740, 21.788, 23.348, 24.161, 25.160, 25.316, 27.360, 27.607, 27.852, 29.212, 29.306, 29.469, 29.523, 32.147, 32.964, 33.140, 33.329, 33.600, 34.378, 34.539, 39.016, 39.415, 39.510, 39.606♦, 42.097, 42.200, 42.298, 42.372, 42.554, 45.217, 45.356, 50.510, 52.723, 55.263, 55.369, 55.553, 55.682, 55.793, 56.508, 56.633, 58.612, 58.849, 61.378, 62.030, 62.188, 62.451, 63.969, 67.830, 70.021, 70.092, 115.773**, 118.090**, 120.438**, 120.526, 122.723**, 131.020, 131.331, 131.411, 136.653, 165.607*, 165.890*, 166.173*, 166.457*, 172.645, 173.877, 173.990, 174.321, 174.420, 175.098, 175.298, 175.435, 175.886, 176.015, 176.123, 176.587, 176.861, 176.919, 177.325, 177.960, 180.256, 180.792 (peaks marked with * and ** indicate TFA COOH, J = 107.1/71.4/35.7 and TFA CF_3, J = 583.0/291.4 respectively; ♦ indicates position of observed ^{13}C label). Additional 2D NMR data as well as pH titration NMR data is reported in Chapter 6-NMR. Confirmed Amino Acid Sequence: see **25**.

NH_2-Cys_{38}-Lys_{39}-Ile_{40}-Val_{41}-Glu_{42}-Met_{43}-Ser_{44}-Thr_{45}-Ser_{46}-Lys_{47}-Thr_{48}-Gly_{49}-Hypusine$_{50}$-His_{51}-Gly_{52}-His_{53}-Ala_{54}-Lys_{55}-Val_{56}-His_{57}-Leu_{58}-OH (27). The terminal FMOC protecting group of fully protected, resin bound **25** (0.101 g peptide/resin; 0.0637 g peptide, 0.0153 mmol) was removed in the usual manner. Fmoc-Cys_{38}(Trt) (0.032g, 0.054 mmol), HBTU (21 mg, 0.054 mmol) and 10 µl of DIEA were added to resin and bubbled for a total of 8 hours. The peptide was then subjected to identical deprotection, cleavage and purification methods described for **25** resulting in a white powder, **27** (3.4 mg, 0.0014 mmol, 9.4% yield). Mass expected: 2378.82, observed: 2379.05 (m/z = +1), 2362.37 (m-OH), 1190.30 (m/z = +2). Purity: <85%. Confirmed Amino Acid Sequence: NH_2-X-K-I-V-G-M-S-T-S-L-T-G-X-H-G-H-A-K-V-H-L-OH (Sequencing was able to report the entire peptide sequence. No amino acid was reported at position 38 and 50 as expected since the automated sequencer does not have cysteine or hypusine as an internal standard). ^{1}H NMR (1.4 mM; 2.0 mg in 0.6 mL 90% H_2O/ 10% D_2O; external NaTSP; pH = 3.12; 7 °C): δ 0.866-0.948 (m, 24 H), 1.156-1.265 (m, 5 H), 1.265-1.552 (m, 10 H), 1.395-1.399 (d, 3H, J = 7.0), 1.552-1.870 (m, 19 H), 1.870-2.099 (m, 7H), 2.099 (s, 3 H), 2.436 (br m, 2 H), 2.525-2.692 (m, 2 H), 2.950-3.057 (br m, 9 H), 3.058-3.360 (m, 9 H), 3.825-4.798 (m, 27 H), 7.270 (s, 1 H), 7.327 (s, 2 H), 7.617 (br s), 8.090 (t, 4 H, J = 14.0, 7.0), 8.354-8.369 (d, 1H, J = 7.0), 8.369-8.581 (m, 5 H), 8.628 (br s, 10 H), 8.794-8.805 (d, 4 H, J = 5.0).

CHAPTER 5
ANALYTICAL–HPLC, TISSUE EXTRACTIONS, AND HPLC-MS

HPLC Fluorescence Assay for Hypusine and Hypusine Dipeptides

The successful preparation of hypusine and hypusine dipeptides described earlier in this dissertation provided sufficient amounts of α-(β-alanyl)-hypusine and α-(γ-aminobutyryl)-hypusine for use as standards in the development of an HPLC fluorescence assay. Sano's group reported the isolation of hypusine and its dipeptides from bovine brain tissue in sub-nanomole/g tissue amounts (Sano et al., 1986, 1987, Ueno et al., 1991). Therefore, an additional objective of this dissertation was to develop an alternative HPLC analytical technique that could detect these compounds at or below the low concentrations previously observed. The successful use of a precolumn derivatization method able to detect hypusine, hypusine dipeptides, and other amino acid standards below 0.5 nmol is described below. Ultimately, this analytical assay was adapted for use in the detection of these compounds in rat brain tissue. This was achieved after ^{3}H-hypusine was used as a tracer in the development a suitable tissue extraction method and the HPLC was coupled to a mass spectrum detection system.

HPLC Experimental

HPLC system setup. The HPLC system (Gilson, Middleton, WI) consisted of two model 306 pumps (A and B), a 401 dilutor, an 805 manometric module, an 811C dynamic mixer, a 231 sample injector, and a Rheodyne injection valve with a 50 µL or 100 µL injection loop. Fluorescence detection measurements were made using a McPherson (Acton, MA) FL-750 HPLC-PLUS spectrofluorescence detector. The

excitation and emission wavelengths were set to 340 nm and 440 nm respectively. A Microsorb-MV C8 (5μ, 100 Å) reversed phase column (25 cm x 4.6 mm ID) from Varian (Walnut Creek, CA) was used in conjunction with a Dynamax C8 (5μ, 5 cm x 4.6mm ID) guard column (Rainin). The column remained at room temperature. Chromatograms were recorded and processed using Dynamax HPLC method manager software version 1.46 (Varian) on a Macintosh computer.

Reagents. β-Alanine (β-ALA) and γ-amino butyric acid (GABA) were obtained from Sigma-Aldrich. α-(β-alanyl)-hypusine (**15**), hypusine (**18**, non-labeled), and α-(γ-aminobutyryl)-hypusine (**16**) were synthesized as described in Chapter 2. HPLC grade acetonitrile, methanol, sodium hydroxide, hydrochloric acid, and water were obtained from Fisher Scientific (Fair Lawn, NJ). *O*-phthaldialdehyde (OPA), 2-mercaptoethanol (2-ME), and *N*-acetyl-L-cysteine (NAC), were obtained from Sigma-Aldrich. The routine use of OPA/2-ME and OPA/NAC (Fig. 5-1) as suitable reagents for the precolumn derivatization of amino acids has been reviewed elsewhere (Alvarez-Coque & Garcia, 1989).

OPA/2-ME. After attempts for the precolumn derivatization of hypusine and its dipeptides with dansyl chloride and fluorescamine reagents proved unsuccessful (data not shown), OPA/2-ME and OPA/NAC reagents were used. The OPA/2-ME reagent was prepared by dissolving 50 mg anhydrous *o*-phthaldialdehyde in 2 mL HPLC grade methanol. To this was added 6 mL of 0.1 M borate buffer (pH 9.4) followed by 100 μL of 2-mercaptoethanol. Brij-35 (100 μL, a surfactant) was also added. The solution was stored in a brown glass vial with a teflon seal and kept at room temperature away from light. The derivatization reagent was filtered through a 0.2 μm frit prior to use.

OPA/NAC. The use of an alternative sulfur moiety was explored when developing a suitable derivatization reagent for the hypusine-containing compounds analyzed. *N*-acetyl-L-cysteine (NAC) was substituted for 2-mercaptoethanol. Solutions of 0.512 g of anhydrous *o*-phthaldialdehyde in 25 mL MeOH (0.153 M) and 1.05 g of NAC dissolved in 50 mL (0.129 M) of sodium borate buffer (0.1 M, pH 9.5) were prepared. The OPA/NAC reagent was prepared by combining 2.5 mL of the OPA solution, 10.0 mL of the NAC solution, and 2.50 μL of 2 N NaOH ([OPA]/[NAC] = 1:3). Reagents were prepared fresh and kept at room temperature in the dark for at least 90 minutes prior to use.

Amino acids and dipeptides. Stock solutions of amino acids and dipeptides were prepared in H_2O with concentrations ranging from 3.50×10^{-4} M to 5.33×10^{-6} M. These concentrations were chosen so that upon derivatization of 25–50 μL of any given stock standard solution, approximately 0.1 to 10 nmol of compound would be derivatized.

Derivatization with OPA/2-ME. The five compounds (β-ALA, GABA, hypusine and both hypusine dipeptides) were each dissolved in HPLC grade water and diluted to a concentration of 1 mM. Each 1 mM standard solution (100 μL) was then combined to form a 0.2 mM standard solution. This 0.2 mM standard (50 μL) was then derivatized with 250 μL of OPA/2-ME reagent (combine, cap, mix 2 minutes). This reaction mixture (50 μL) was then injected onto the HPLC column. Each peak that eluted corresponded to 10 nmol of compound derivatized. This process was repeated with 25, 10, 3.75, and 1.50 μL aliquots of 0.2 mM standards (5, 2, 0.75, and 0.3 nmol respectively) to determine if the fluorescent peak area observed was linear with

concentration. Reactions times were kept to five minutes or less from addition of fluorescent reagent to injection onto column.

Derivatization with OPA/NAC. Typically, 25 µL of a hypusine/hypusine-dipeptide standard stock solution was mixed with 150 µL of OPA/NAC reagent and vortexed. Reaction times of 1, 2, 5, 7, and 30 minutes were tested to determine the optimum reaction time for OPA/NAC derivatization. A reaction time of one minute gave the maximum fluorescence intensity (Fig. 5-5). After each reaction time, the reaction was quenched via the addition of 250 µL of NaAcetate buffer (50 mM, pH 7.5) and then immediately injected onto the column (100 µL injection loop). The advantages to this reagent were the lack of powerful stench (caused by 2-ME) and the apparent increased stability of the derivatized compound, which resulted in more intense fluorescent peak areas.

Mobile phase solvent conditions. Solvent A, 50 mM sodium acetate buffer, was prepared by dissolving 4.12 g sodium acetate (82.03 g/mol) in 1000 mL of HPLC grade water and adjusting to pH 7.2 using dilute aqueous acetic acid. To this was added 100 mL acetonitrile. Solvent B was prepared by mixing acetonitrile and water in a ratio of 70:30 (v/v). Mobile phases were simultaneously degassed and vacuum filtered through a 0.45 µm nylon membrane (MSI, Westboro, MA) using a mobile phase filtration/delivery cap (Kontes).

Mobile phase gradient for derivatization with OPA/2ME. A flow rate of 1.0 mL/min was used throughout each sample run. Each run began with a two minute hold at 18% B followed by a 23 minute ramp to 50% B. This was followed by a three minute ramp to 100% B, a nine minute hold at 100% B, and then a two minute ramp back down

to 18% B. At least a 10 minute equilibration period was allowed before the injection of the next sample.

Mobile phase gradient for derivatization with OPA/NAC. A flow rate of 1.0 mL/min was used throughout each sample run. Each run began with a two minute hold at 5% B followed by a 22 minute ramp to 32% B. This was followed by a six minute ramp to 100% B, an eight minute hold at 100% B, and then a two minute ramp back down to 5% B. At least a 10 minute equilibration period was allowed before the injection of the next sample.

As can be seen from the change in the percentage of starting mobile phase, the OPA/NAC derivatives required a more highly aqueous environment to be properly retained and separated on the C8 column. When the mobile phase gradient described for OPA/2ME reagent was attempted with OPA/NAC derivatives, the compounds washed off the column in less than eight minutes with almost no separation. Thus, the method was modified accordingly.

HPLC Results

The elution order for the compounds in the five-compound standard derivatized with OPA/2-ME was as follows: β-ALA, GABA, β-ALA-hypusine, hypusine, and GABA-hypusine. The dipeptides and hypusine eluted in the same order listed above when the alternative reagent OPA/NAC was used (Fig. 5-2a). All ranges and standards tested displayed a linear relationship between fluorescence and concentration and yielded an R^2 value of 0.996 or greater (Fig. 5-2b).

The OPA/NAC reagent was found to be superior for the HPLC analysis of the two dipeptides. First, the fluorescence peak area observed for the derivatized dipeptides was

four to five times greater with OPA/NAC than for OPA/2-ME (Fig. 5-3a). This apparent increase, possibly due to a more stable derivatize, allowed a lower limit of detection. A more stable derivative would maintain its fluorescence quality longer and remain intact during extended HPLC elution conditions. Indeed, the ~ 0.1 nmol dipeptide standard was easily observed when OPA/NAC was used. In contrast, the 0.1 nmol dipeptide standard could not be distinguished from baseline noise when reacted with OPA/2ME reagent. Also, utilizing a reaction time of one minute gave the maximum fluorescence intensity (as seen in Fig. 5-3b with amino acid standards). The second advantage for the use of OPA/NAC vs. OPA/2ME was the elimination of the use of 2-mercaptoethanol, which is well known for its pungent odor. Therefore, the use of a fluorescent tag such as OPA/NAC is an effective means for detecting and separating the α-(β-alanyl)-hypusine and α-(γ-aminobutyryl)-hypusine dipeptides and hypusine via precolumn derivatization and HPLC. The methods described here were further adopted for the detection of these compounds in biological samples such as rat brain tissue. This method could also be easily modified for the analysis of similar small molecular weight compounds that lack an inherent fluorophore.

Use of ^{3}H-Hypusine as a Tracer for Tissue Extraction Method Development

The successful synthesis of a radiolabeled hypusine compound allowed access to a previously unavailable tracer molecule. A known amount of ^{3}H-hypusine could now be added to brain homogenates and a procedure to extract the entire tracer could be developed. If successful, this extraction method could be used for the assay of free hypusine and its dipeptides in rat brain tissues.

^{3}H-Hypusine tracer experiment. The brains (cerebrum) of five male Harlan Sprague Dawley rats were carefully excised from the euthanized animals (7.13 g total weight). Approximately 1.01 μCi (1.6 μL, 2.24 x 10^6 DPM) of ^{3}H-hypusine (**18**) were mixed with 3.5 mL sterile saline (0.9% NaCl) and added to the brain tissue during homogenization in a glass homogenization tube. The homogenization tube was kept in an ice bath during the procedure. The entire homogenate was transferred to a tared 250 mL centrifuge bottle. The homogenization tube was rinsed with both saline and methanol and transferred to the bottle containing the homogenate. The total volume of the mixture was 175 mL (92% MeOH). The brain homogenate was then centrifuged (2790 rpm, 25 min, 5 °C). The aqueous methanol solution was decanted and concentrated via rotovap. A beige mixture remained and was transferred to a vial. The flask was washed with 1:1 MeOH/H_2O and transferred to vials as well. Scintillation counting of the methanol extract and portions of the digested pellet determined that 80% of the tritium radioactivity remained in the brain pellet. (Brain pellet samples were digested by adding 200 μL 1 N NaOH to an 11.5 mg pellet portion, vortexing and then placing in a 60 °C water bath for one hour. Afterwards, the samples were allowed to cool to room temperature. Then 300 μL of 1 N HCl was added to neutralize the sample. The entire mixture was transferred to a scintillation vial. Scintillation fluid (10 mL) was added, the vial was vortexed, and after five minutes, the solution was counted).

Choice of solvent for tissue pellet extraction. After MeOH proved to be ineffective for the extraction of ^{3}H-hypusine from rat brain homogenates, several solvents were prepared in order to attempt the extraction of ^{3}H-hypusine from the brain pellet. The goal was to identify a solvent that would extract the majority of the radioactivity (i.e.

^{3}H-hypusine) from the tissue in the minimum number and volume of extractions. The following solvents were tested: (1) tributylamine, (2) *N,N*-dimethylbenzylamine, (3) dicyclohexylamine, (4) 5% dicyclohexylamine in MeOH, (5) 5% tributylamine in MeOH, (6) 5% triethylamine in MeOH, (7) 0.5 M Sodium Barbital buffer (pH = 10.22), and (8) 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH = 10.68). In general three pellet portions (100-150 mg each) were sampled from the stock, ^{3}H-hypusine-containing pellet for each solvent tested. Two pellet samples were subjected to a series of extractions with a particular solvent. The extracts were then counted via scintillation methods and compared to the total counts observed in the third pellet (^{3}H-hypusine-containing, non-extracted) after digestion.

Results of solvent choice experiment. Initially, the 5% solutions of basic amines in MeOH extracted a majority of the tracer compound (> 75%), particularly 5% dicyclohexylamine in MeOH, but posed the problem of interfering with the HPLC derivatization method developed earlier (i.e. large amounts of free amine from extraction solvent masked small amounts of free amines/dipeptides being derivatized). Extractions with chilled 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH = 10.68) had the highest yields—greater than 90%. This high yield along with the absence of any extraneous free amine moieties that would interfere with HPLC analysis made this buffer the solvent of choice for future tissue extractions.

Protein clean up. To minimize the amounts of proteins and other large particles extracted from brain tissue homogenates, tissue extracts were filtered through a G-25 Sephadex column. A slurry of G-25 Sephadex in 0.05 M NaOAc (pH 7.5) was prepared and a small column was poured (18 cm x 1.7 cm i.d.). Blue dextran (M.W. 2 x 10^6) was

added to the column and eluted with the sodium acetate buffer to determine the void volume of the column (fractions monitored OD, $\lambda = 260$ nm). Phenol red (phenolsulfonephthalein, sodium salt M.W. 376.36) was added to the column as an additional marker and eluted (fractions monitored OD, $\lambda = 432$ nm). ^{3}H-hypusine (14 µL in 100 µL of buffer, 1.2×10^5 DPM total) was then eluted through the column. Fractions were collected and counted by scintillation to determine where the radioactive compound eluted. It was determined that ^{3}H-hypusine eluted post void volume and prior to the phenol red marker (Fig. 5-4a).

To determine where hypusine and related compounds would elute through such a column after extraction from tissue, a small portion of the brain pellet (0.4654 g) containing the ^{3}H-hypusine tracer was extracted with 0.1 M $Na_2CO_3/NaHCO_3$ as follows: 2 x 500 µL of buffer was added to the pellet in a 15 mL centrifuged tube. The pellet/buffer was then vortexed for 60 seconds and centrifuged at 3000 rpm for 10 min at 10 °C. The supernatant was collected and 2 x 100 µL were analyzed via liquid scintillation counting. CPM "counts" were corrected for background radioactivity, averaged, and converted to total DPM/mL. The pellet was extracted with a total of 5 mL buffer. Two additional brain pellet samples (139.0 mg and 151.2 mg respectively) containing the tracer compound were digested in 300 µL 1 N NaOH for three hours at 60 °C. After cooling to room temperature, the digests were neutralized with 300 µL 1 N HCl. Each digest was dissolved in 10 mL of scintillation cocktail fluid and counted. The average total DPM present in the brain pellet was calculated to be 2.30×10^5 DPM. The first three extractions removed 92 % of the total DPM. The three 1 mL extracts were added to a G-25 Sephadex column and eluted with 0.05 M NaOAc (pH 7.5). Fractions

were collected in 1 mL portions and counted by scintillation (100 µL samples from each fraction). Fractions were also subjected to a Lowry protein determination assay (procedure reviewed elsewhere, Peterson, 1979; Waterburgh & Matthews, 1994) in order to assess the retention times of large proteins eluting through the column. Having large molecular weights, it was expected that proteins would elute in or near the void volume (< 15 mL). ^{3}H-hypusine eluted after the protein containing fractions (Fig. 5-4b). Therefore, it was determined that the extraction of rat brain tissue (non-radioactive) with 0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.68) followed by filtration through a G-25 Sephadex column with 0.05 M NaOAc (pH 7.5) should effectively separate free hypusine and related compounds from larger proteins and biomolecules with minimal overlap. Analysis of these fractions by the OPA/NAC fluorescence assay described above should allow the detection of these compounds if they are indeed present in the tissue extracts at concentrations at or above 0.1 nmol/g (wet weight, rat brain tissue).

Extraction and HPLC Analysis of Rat Brain Tissue for Hypusine and Dipeptides

The assays described above were then applied to rat brain homogenates that did not contain a ^{3}H-hypusine tracer. Two separate experiments were carried out as described below.

Experiment 1. The brains of 10 female Wistar rats (14.9 g total weight) were rinsed with 0.9% NaCl and then homogenized in 0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.7) in a 250 mL plastic centrifuge bottle (held in ice bath). The homogenate was shaken vigorously for 30 seconds and then centrifuged for 20 min at 3000 rpm, 10 °C. The supernatant was decanted into a tared round bottom flask. The extraction process was repeated with 4 x 50 mL of buffer. The total volume of supernatant extract solution was

600 mL. The pH of the extract was adjusted to 7.0 with cold, concentrated acetic acid added dropwise (~ 1 mL total). The supernatant solution was divided into three equal portions and lyophilized. Portions of the dried powder were then redissolved in sodium acetate buffer (0.05 M, pH 7.5) and eluted through a G-25 Sephadex column. Fractions were collected and grouped into the following categories: (1) "void volume" fraction, (2) post-void #1, (3) post-void #2, and (4) post-void #3. Fractions were pooled according to the categories listed and lyophilized to obtain dry powders. Approximately 10% of each lyophilized fraction category was then dissolved in water and analyzed by the HPLC OPA/NAC method described earlier. Peaks were off scale for the "void volume" fraction due to protein and large molecule interference. Analysis of the post-void #2 and #3 fraction yielded fairly vacant chromatograms. However, analysis of post-void #1, the fraction expected to contain hypusine and related compounds contained several suspect peaks (Fig. 5-5).

Therefore, portions of the lyophilized, purified extract were combined with ~ 1 nmol each of a hypusine, β-ALA-hypusine, and GABA-hypusine standard respectively. Hypusine and GABA-hypusine were observed to coelute with two of the peaks in the "suspect region" of the HPLC chromatogram (Fig. 5-6). β-ALA-hypusine eluted between two of the other peaks in the suspect region of the chromatogram. The standard curves for these compounds were then used to determine the concentrations of the suspected hypusine and GABA-hypusine peaks as 2.4 nmol/g and 22.4 nmol/g brain tissue respectively. These values agreed with those previously published—hypusine, 1-8 nmol/g and GABA-hypusine, ~1 nmol/g (Sano et al., 1986, 1987; Ueno et al., 1991).

Additional LCMS experiments discussed below confirmed the presence of hypusine in the tissue extracts. The dipeptides were not detected.

Experiment 2. The experiment above was repeated to observe reproducibility with six additional rat brains (8.0 g total weight). Again, several peaks were observed in the region of the HPLC chromatogram where the hypusine and hypusine dipeptide standards elute during the OPA/NAC method. Purified extracts were again combined with approximately 1-nmol standards of hypusine, β-ALA-hypusine, and GABA-hypusine. Again, hypusine and GABA-hypusine were observed to coelute with two of the peaks in the "suspect region" of the HPLC chromatogram whereas the β-ALA-hypusine standard did not coelute with any of the peaks in the suspect region. The standard curves for these compounds were used to determine the concentrations of the hypusine and GABA-hypusine suspect peaks as 3.34 nmol/g and 27.0 nmol/g brain tissue respectively.

It should be noted that the sole fact that peaks present in rat brain tissue extract were observed to coelute with hypusine and GABA-hypusine standards does not definitively prove nor disprove the presence of these compounds in their free form in normal brain tissue. Further analyses of these "suspects" are required and are discussed in the next section. However, it is encouraging that the concentrations of the peaks detected fell within the same order of magnitude as those previously published for the standards more than 10 years ago using an alternate extraction method.

HPLC-MS Analysis of Hypusine, Hypusine Dipeptides, and Samples from the Extracts of the Rat Brain Homogenate

Experiments such as HPLC coupled to mass spectrometry could confirm the mass of the derivatized suspect peaks and compare them to the mass obtained for the

derivatized standard peaks. As the suspect peaks eluted from the HPLC column, they would be directly injected into a mass spectrometer. Pure standards, derivatized prior to injection onto the HPLC column, could provide a "fingerprint" spectrum by which to identify such compounds in the extracts of tissue samples. Such an experiment could definitively confirm the presence of free hypusine and hypusine dipeptides present in the extracts of rat brain homogenate. The addition of mass spectrometry to the precolumn fluorescence derivatization HPLC method would also allow us to determine the type and structure of derivative being formed by the OPA/NAC reagent (mono- vs. di-derivatize due to presence of two primary amines in each compound). The reaction mechanism for the derivatization of free amines by OPA/2ME (Alvarez-Coque & Garcia, 1989) along with the HPLC-MS data detailed below would allow for structural determination of the derivatized hypusine-containing compounds. Fortuitously, Dr. Jodie V. Johnson, Ph.D. of the Chemistry department at UF graciously agreed to help with the completion of this experiment.

HPLC-MS Experimental. A ThermoFinnigan LCQ Mass Spectrometer (San Jose, CA) was coupled to a Beckman Instruments System Gold (Fullerton, CA) HPLC system with model 126 pump. The mass spectrometer was run in electrospray ionization mode (ESI) with N_2 as the sheath gas. The spray voltage was 3.5 kV with a cap temperature of 250 °C. The cap voltage and tube lens offset were set to 15 and 0 V respectively. A Phenomenex (Torrance, CA) LUNA C18 guard column connected to a Waters (Milford, MA) Symmetry Shield RP18 2.1 x 150 mm column were used. Mobile phase A was 9.9 mM NH_4OAc in H_2O. Mobile phase B was 100% MeOH. Both mobile phase solutions were filtered and degassed prior to use. The flow rate was set to 0.15

mL/min. The HPLC gradient was 100 %A at t = 0 minutes to 5%A:95%B at t = 45 minutes. A 100 μL injection loop was used to deliver samples onto the HPLC/MS system. In order to assist the ionization of the analytes, the mobile phases were modified post-column with the addition of 1% HOAc in MeOH via a second pump (Applied Biosystems model 400 solvent delivery system) and a PEEK Tee-union located immediately after the UV detector and before the ESI source for the mass spectrometer. The flow rate for this second pump was 0.1 mL/min. The UV detector was an Applied Biosystems Model 785A Programmable Absorbance Detector with wavelength set to 340 nm.

Sample preparation. Samples were derivatized for analysis on the HPLC-MS system in a manner identical to the pre-column OPA/NAC derivatization method described above. A reagent blank and a three-compound standard mixture (hypusine, 4.0 x 10^{-5}M; β-alanyl-hypusine, 4.1 x 10^{-5}M; and GABA-hypusine, 1.3 x 10^{-5}M) were prepared for analysis.

HPLC-MS sample analysis. Initially, a reagent blank was run on the HPLC/MS system to obtain a baseline chromatogram and identify any UV and MS peaks that were a direct result of the derivatization reagents themselves. Next, a mixture of hypusine, β-alanyl-hypusine, and GABA-hypusine standards was analyzed via a reverse phase gradient C18 HPLC/UV/(+)ESI-MS and MS/MS routines. A 100μL sample injection corresponded to 235 pmol on column for hypusine and β-alanyl-hypusine and 76 pmol on column for GABA-hypusine. A fingerprint mass spectrum was obtained for the three standards. Next a tissue extract sample from the 10-brain homogenate extract (which produced the "suspect" peaks in the original OPA/NAC HPLC experiments discussed

earlier) was analyzed. Finally, a sample from the 10-brain homogenate extract was mixed with a known concentration of the three-compound standard and analyzed.

HPLC-MS Results and Discussion

OPA/NAC precolumn derivatization and HPLC-MS analysis of hypusine and hypusine-dipeptide standards provided a slightly different elution pattern for the standards. The order of elution was slightly different from that observed on the HPLC system alone–Hypusine (34.83 min), β-alanyl-hypusine (35.92 min), and GABA-hypusine (37.93 min). Figure 5-7 shows the UV trace for the three-standard mixture. Recall that hypusine eluted between the two dipeptides on the original HPLC system. This different order of retention and extended retention time was the result of the HPLC-MS system utilizing a different HPLC column (C18 vs. C8), flow rate conditions (0.15 mL/min vs. 1.0 mL/min), and solvent conditions (see experimental for HPLC and HPLC-MS). The mass peaks observed for each of the standards along with their MS/MS fragmentation patterns corresponded to the formation of a di-derivative (Fig. 5-7, 5-9). In other words, the OPA/NAC derivatization method placed fluorescent moieties on the two primary amines located on each compound. The $[M+H]^+$ mass peaks observed for each compound were m/z = 756, 827, and 841 respectively. This corresponds to the molecular weight of each compound plus two OPA/NAC moieties on the primary amines of each compound. Previously, it was not known whether a mono or di-derivative was formed with these compounds under the conditions of these experiments. Hypusine was previously observed to form both mono- and then di-derivatives using dansylchloride (Bergeron et al., 1998b). Due to the large excess of OPA/NAC reagent used compared to the standards, it was suspected that a di-derivative compound would be formed. The

LCMS analysis was able to confirm this hypothesis. The only sample-related UV peaks observed in the chromatograms are those indicated for the di-derivatives of the three standards (Fig. 5-7). There were no obvious mono-derivatives detected.

Based on the mechanism proposed for the formation of an OPA/NAC derivative, the fragmentation patterns observed are consistent with a di-derivatized compound for the three standards analyzed (Fig 5-10). The structures of the possible fragmentation occurring for the GABA-hypusine compound are shown in Figure 5-11 and discussed here. A loss of 129 from the initial $[M+H]^+$ m/z of 841 to give an m/z of 712 indicates the loss of a portion of the OPA/NAC moiety, particularly the acetyl and carboxylic acid groups. Alternatively, a loss of 163 from the parent ion corresponds to a complete loss of the N-acetyl-cysteine group to give an m/z of 678. An additional loss of 163 mass units from the 712-ion indicates the removal of the aromatic OPA moiety to give an m/z of 549. A loss of 161 from the 678-ion indicates the removal of the second N-acetyl-cysteine group to give an m/z of 517. Similar patterns of fragmentation were observed for hypusine (Table 5-1, Fig. 5-8, 5-9) and β-alanyl-hypusine standards as well (Fig. 5-9).

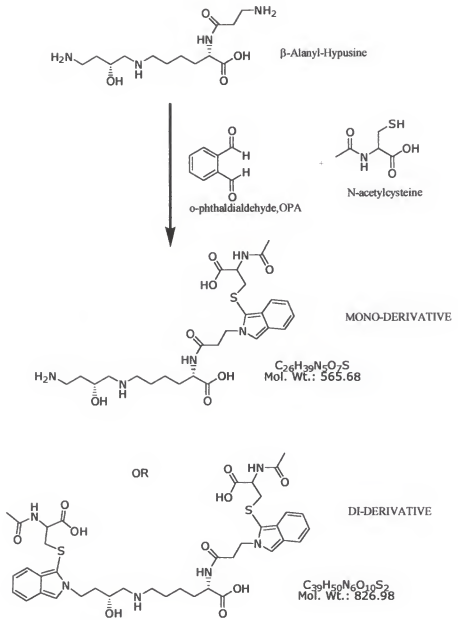
Analysis of the rat brain extract sample identified peaks containing masses corresponding to both hypusine and the GABA-hypusine compound. The retention time (34.38 min) and mass spectra for the hypusine suspect peak correlated well with the hypusine standard (Fig. 5-12). However, only the mass spectra for the GABA-hypusine peak appeared to agree with the standard (Fig. 5-13). The retention time of 35.36 min for the suspect was nearly three minutes earlier than that observed for the GABA-hypusine standard. This peak was at first a suspect for β-Ala-Hypusine due to the retention time being quite close to it (β-Ala-Hyp standard, 35.92 min). However, as stated, the mass

peaks matched the GABA-standard more closely. A sample of the rat brain extract sample was then mixed with the three standards, derivatized with OPA/NAC as described above, and analyzed by LCMS. The standard for hypusine was observed to coelute with the peak suspected to be free hypusine in the rat brain extract (Fig. 5-14, 5-15). The GABA-hypusine standard eluted after the peak that was initially suspected to be free GABA-hypusine (Fig. 5-14). No peaks (UV or MS) in the rat brain extract corresponded to the β-alanyl-hypusine compound.

It can therefore be concluded that only free hypusine was observed in the rat brain extracts by the analytical methods presented here. A comparison of the relative peak areas of the di-derivatized hypusine ion-peaks in the standard, brain extract, and "spiked" brain extract is displayed in Table 5-1. The value for relative abundance and retention times for the main ion peaks observed are in good agreement with each other. Based on the standard curve for hypusine in this study (Fig. 5-2), the amount of free hypusine in the rat brain extracts observed was calculated to be approximately 2.40 to 4.40 nmol/g brain tissue (using area of suspect peak "3" in Fig. 5-5). This value is within the range of previously published values for the concentration of hypusine in tissue extracts (approximately 1-8 nmol/g). The dipeptides were not identified in tissue extracts by the methods described here.

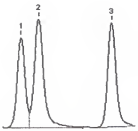
Table 5-1. Comparison of Relative Peak Areas for OPA/NAC Derivatized Hypusine

		Standard		Brain Extract		Brain Extract + Spike	
Ion	Hyp-2	Peak Area	Rel Abund	Peak Area	Rel Abund	Peak Area	Rel Abund
Retention Time		34.83		34.38		35.04	
[M+H-CO2]+	712						
[MH-163]+	593	56288297	88.42	663862	75.48	2911679	95.48
[712-163]+	549	63657711	100.00	879554	100.00	3049445	100.00
[549-129]+	420	25942468	40.75	392630	44.64	1380744	45.28



The OPA/NAC reagent reacts with free primary amines (β-Ala-hypusine shown here) to form either a mono- or di-derivatives with these proposed structures.

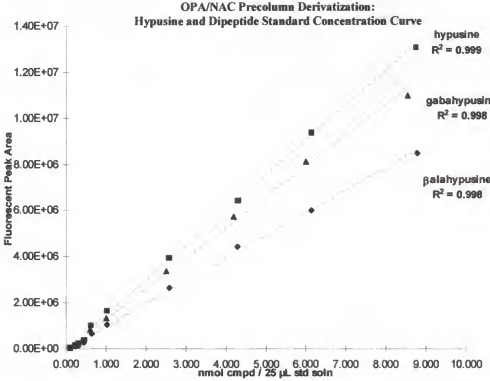
Figure 5-1. OPA/NAC Derivatization of Primary Amines



Peak	Ret. Time	Type	Height	Area
1	16.111	*M1	250875	2385470
2	16.426	*M2	303238	3481844
3	17.740	*M	289896	2993078

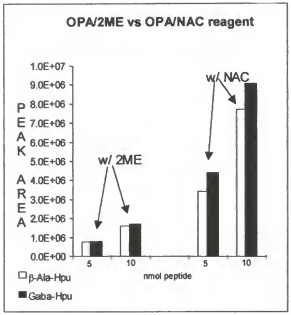
OPA/NAC derivatized β-ALA-hypusine (1), hypusine (2), and GABA-hypusine (3) standards (2.5 nmol each)

A- Sample Chromatogram of Hypusine, β-Ala-Hypusine, and GABA-Hypusine

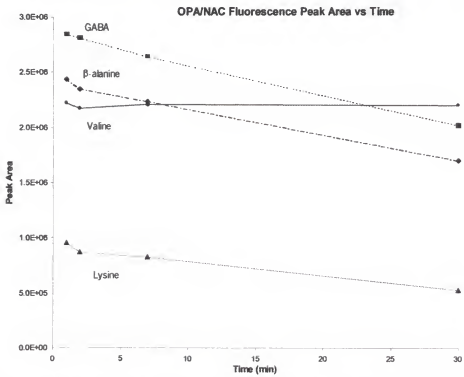


B- Standard Concentration Curve for Hypusine, β-Ala-Hypusine, and GABA-Hypusine--linear from 0.1 to ~ 9.0 nmol compound derivatized

Figure 5-2. Pre-Column Derivatization of Hypusine, β-Ala-Hypusine, and GABA-Hypusine–A) Sample HPLC Chromatogram and B) Standard Curve

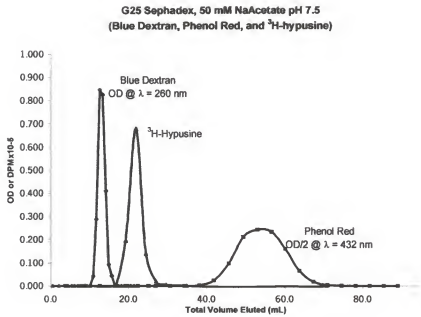


A- Comparison of the fluorescent intensity of the dipeptide peaks when 2ME or NAC was used as the sulfur moiety in the OPA reagents.

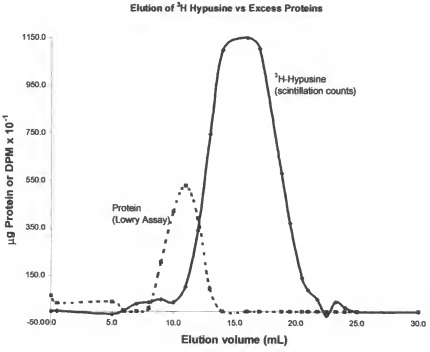


B- Time Course Comparison – OPA/NAC reagent gives the greatest peak fluorescence intensity when reaction times are kept to 1 minute followed by immediate injection onto HPLC.

Figure 5-3. Comparing the Reagents–A) OPA/2ME vs. OPA/NAC and B) Time Course of OPA/NAC Reagent

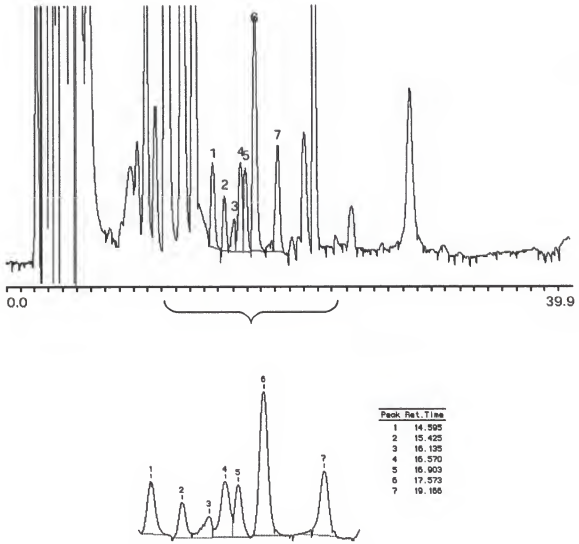

G25 Sephadex, 50 mM NaAcetate pH 7.5
(Blue Dextran, Phenol Red, and 3H-hypusine)
Blue Dextran
OD @ λ = 260 nm
3H-Hypusine
Phenol Red
OD/2 @ λ = 432 nm
OD or DPMx10-6
Total Volume Eluted (mL)

A- Elution of ^{3}H-hypusine in relation to "visible" marker compounds, Blue Dextran and Phenol Red—Data on y-axis is scaled so that both absorbance (OD) and DPM can be observed simultaneously



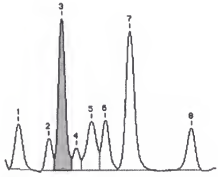
B- Elution of ^{3}H-hypusine in relation to excess proteins from tracer-containing rat brain extract sample

Figure 5-4. Elution of ^{3}H-Hypusine through G-25 Sephadex in Relation to A) Blue Dextran and Phenol Red and B) Excess Protein from Tissue Extract



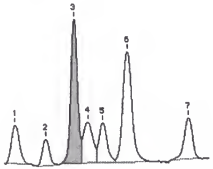
Expansion of "suspect region" of rat brain extract

Figure 5-5. HPLC Chromatogram of Rat Brain Extract–"Suspect Region"



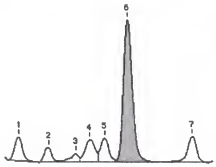
Peak	Ret.Time
1	14.580
2	15.376
3	15.730
4	16.103
5	16.518
6	16.863
7	17.528
8	19.146

Rat brain extract with 1 nmol β-ALA-hypusine spike



Peak	Ret.Time
1	14.565
2	15.388
3	16.126
4	16.495
5	16.898
6	17.528
7	19.150

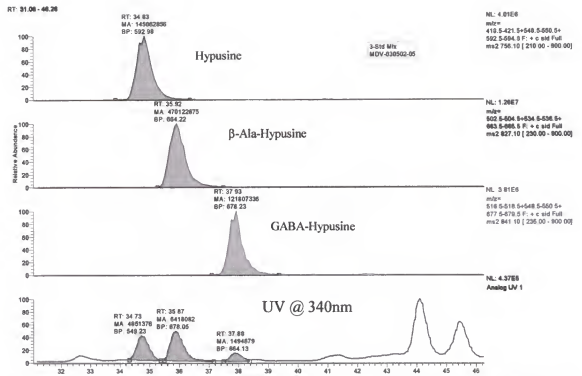
Rat brain extract with 1 nmol hypusine spike



Peak	Ret.Time
1	14.483
2	15.283
3	16.001
4	16.378
5	16.766
6	17.366
7	19.085

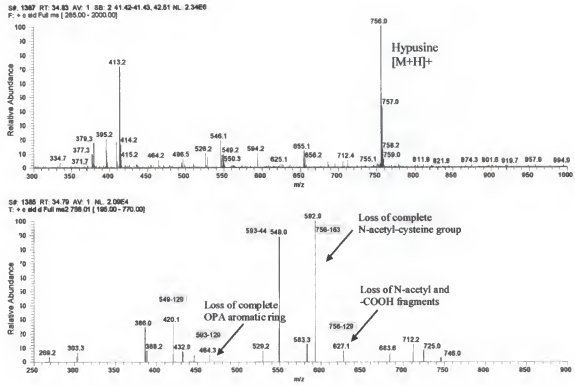
Rat brain extract with 1 nmol GABA-hypusine spike

Figure 5-6. HPLC Chromatogram of Rat Brain Extract Mixed with Standards (a.k.a "Spike Experiment")



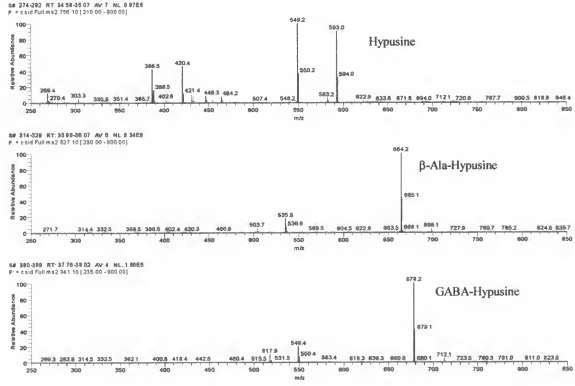
3-standard mixture. The only sample-related UV peaks are those indicated for the di-derivatives of the three standards (bottom chromatogram). There were no obvious mono-derivatives detected. The slight difference in retention time between UV and MS traces—for example, 34.73 min vs. 34.83 min--is a result of the sample eluting through the UV detector first and MS detector second. The top three traces for the standards represent of region of the MS spectra representing the prominent base peak (BP) for each compound (m/z= 593, 664, and 678 for hypusine, β-ala-hypusine, and GABA-hypusine respectively).

Figure 5-7. HPLC-MS–UV Chromatogram of OPA/NAC Di-Derivatized Hypusine, β-Ala-Hypusine, and GABA-Hypusine



The (+)ESI-MS is dominated by the m/z 756 [M+H]+ ion (top) which readily underwent CID to produce the daughter spectra (bottom) containing ions due to loss of the pieces of the derivative (129u and 163 u). The di-derivatives of the β–Ala- and GABA-Hypusine also yielded predominantly their [M+H]+ ions (m/z 827 and 841 respectively–data not shown).

Figure 5-8. HPLC-MS–(+)ESI-MS of Hypusine after OPA/NAC Precolumn Derivatization



The (+)ESI-CID-MS/MS of the [M+H]+ ions of the di-derivatives of each of the analytes of interest. The main peak for each compound (593, 664, and 678) indicates a loss of 163 that corresponds to the removal of the N-acetyl-cysteine group from the parent ion.

Figure 5-9. HPLC-MS–(+)ESI-CID-MS/MS of the [M+H]+ ions of the Hypusine and Dipeptide Standards

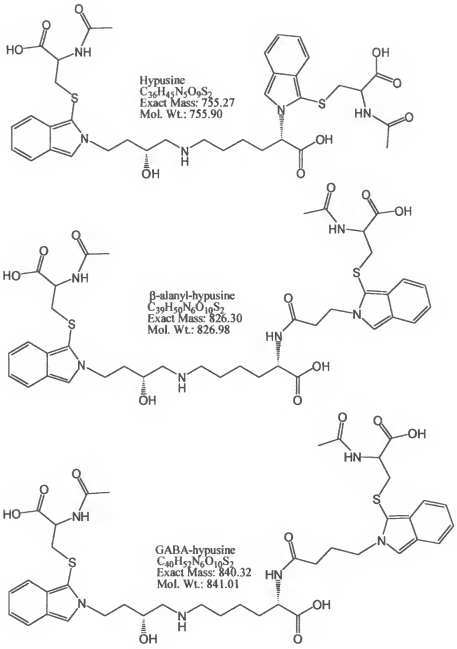
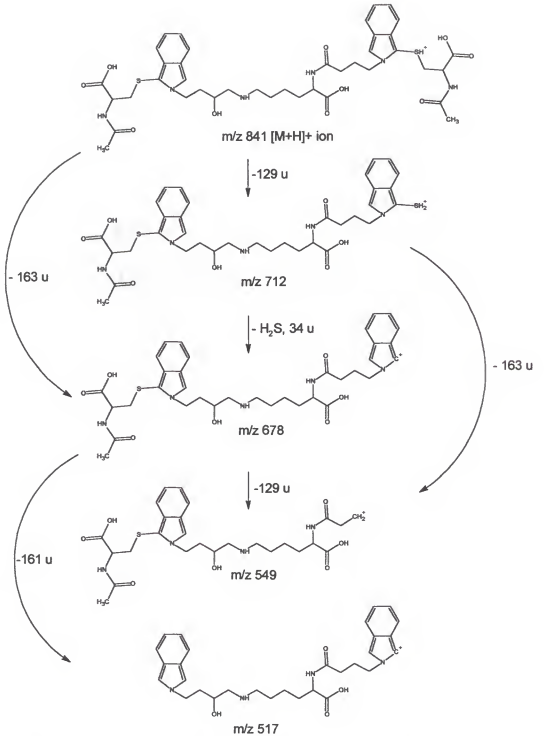


Figure 5-10. Proposed Structures of OPA/NAC Di-Derivatized Compounds after LCMS



Possible (+)ESI-CID-MS/MS Fragmentation Pathway for the m/z 841 [M+H]+ ion of GABA-Hypusine-di-derivative. Similar Fragmentation Pathways were observed for Hypusine and β-Ala-Hypusine as well.

Figure 5-11. Possible Fragmentation Pathway for Di-Derivatized GABA-Hypusine

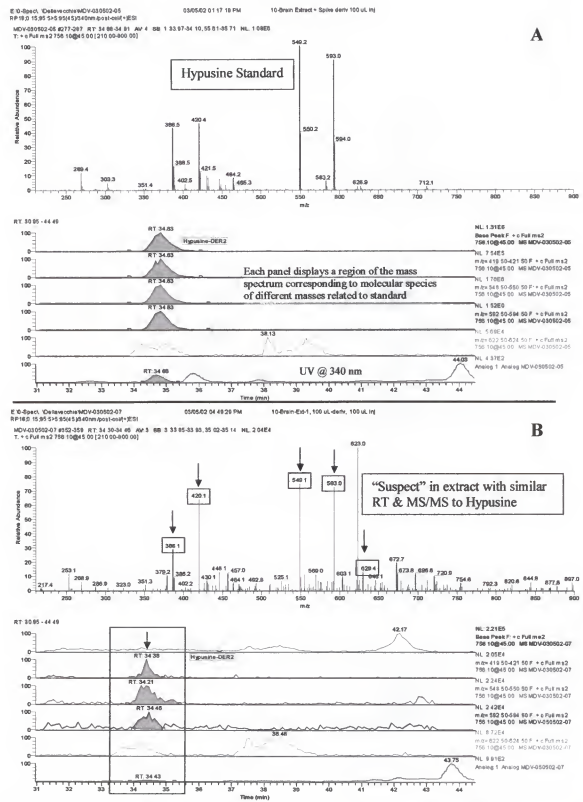


Figure 5-12. Comparison of HPLC-MS Data of A) Hypusine Standard and B) Rat Brain Extract from Experiment #1 Containing Hypusine "Suspect"

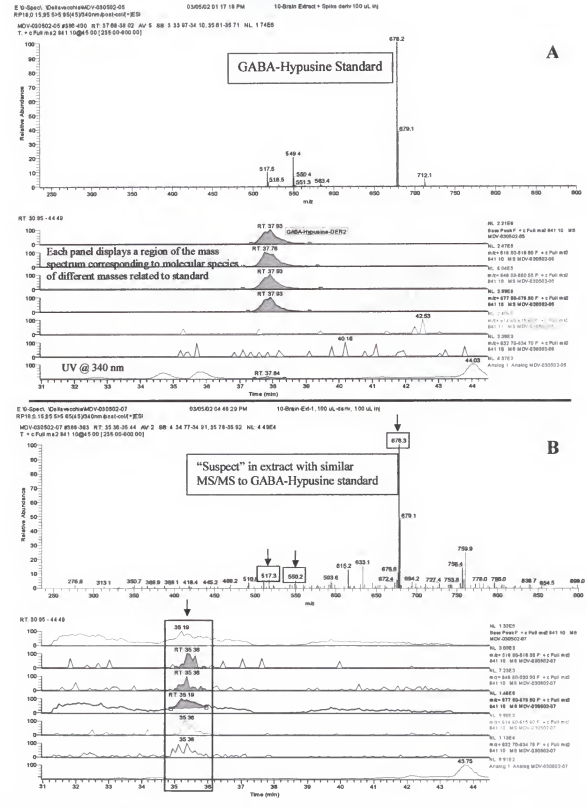
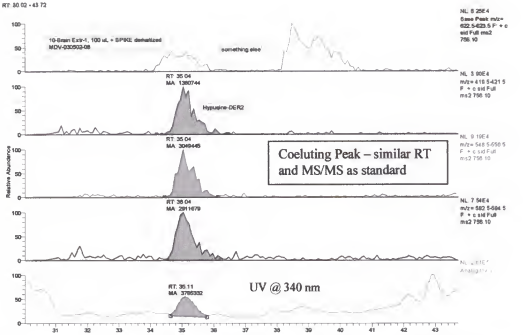
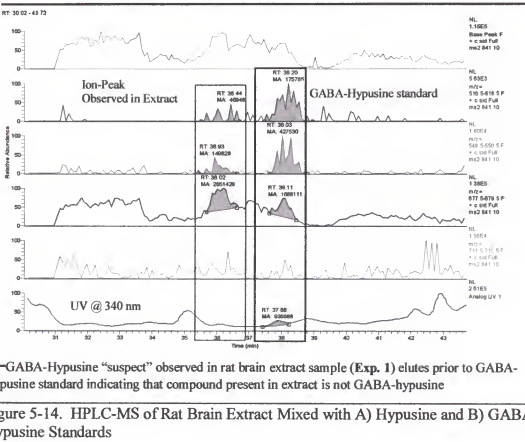


Figure 5-13. Comparison of HPLC-MS Data of A) GABA-Hypusine Standard and B) Rat Brain Extract from Experiment #1 Containing GABA-Hypusine "Suspect"

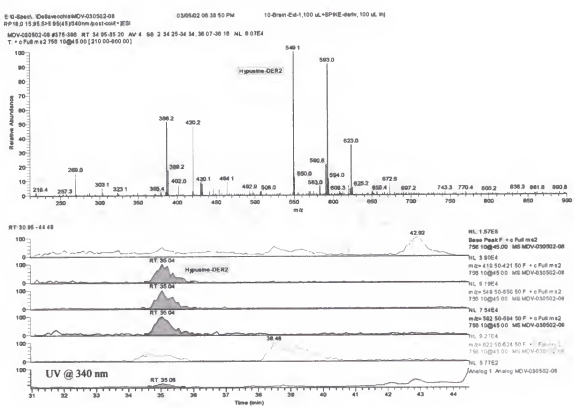


A–Hypusine "suspect" observed in rat brain extract sample (**Exp. 1**) coelutes with Hypusine standard



B–GABA-Hypusine "suspect" observed in rat brain extract sample (**Exp. 1**) elutes prior to GABA-Hypusine standard indicating that compound present in extract is not GABA-hypusine

Figure 5-14. HPLC-MS of Rat Brain Extract Mixed with A) Hypusine and B) GABA-Hypusine Standards



The matching MS daughter ion patterns observed in the hypusine standard (Fig. 5-12a), brain extract hypusine "suspect" (Fig. 5-12b), and standard co-injected with extract (Fig. 5-14a, 5-15) are evidence for the presence of hypusine in the rat brain extracts.

Using the HPLC standard curve data (Fig. 5-2b) and the MS data presented above, peak 3 of the suspect region shown in Figure 5-5 can be confirmed as Hypusine with a concentration of 2.40-4.40 nmol/g (wet weight rat brain).

Figure 5-15. HPLC-MS of Rat Brain Extract with Hypusine Standard

CHAPTER 6
NMR–ADDITIONAL ANALYSES OF EIF-5A PEPTIDE FRAGMENTS

General

Organic chemists routinely prepare samples for analysis by nuclear magnetic resonance (NMR) in order to assess the purity and structural integrity of the compounds they have diligently synthesized. Chemical shifts, splitting, and coupling constants are recorded and compared to a theoretical "spectrum" that is expected for the compound based on the synthesis performed to obtain it. This data is often used to verify the success or failure of a synthesis and to compare results obtained by different researchers. Initially, these techniques were only reliable for small molecules with simple structures. With the availability of high-field superconducting magnets, NMR can routinely be used to analyze more complicated compounds with large molecular weights (Reid et al., 1997).

The routine one-dimensional NMR experiment involves placing a sample into a commercially available NMR tube (typically 5 mm, i.d.), dissolving the sample in a deuterated or partially deuterated solvent, lowering the sample into a strong magnetic field, radiating the sample with a radio-frequency pulse, and recording the response of the sample. Chemical shifts of the various peaks observed in an NMR spectrum are typically referenced to internal or external standards such as tetramethylsilane (TMS, set to 0 ppm) or 3-trimethylsilylpropionate (TSP, set to 0 ppm) respectively. It is from references such as these that the chemical shift assignments for atoms in a particular compound (typically ^{1}H or ^{13}C) are based.

The positions of resonances in an NMR spectrum are sensitive to solvent conditions, temperature, and pH. Chemical shifts can also be affected by the neighboring nuclei in a molecule. Protons, for example, are shielded by the valence electrons that surround them. The electronic environment of different nuclei have varying abilities to shield themselves from the magnetic field in an NMR system. This causes nuclei to resonate at different frequencies causing different chemical shifts as well (Hinds & Norton, 1994). These properties allow the chemist to distinguish aliphatic or methyl group protons from protons next to heteroatoms like nitrogen or oxygen in an NMR spectrum. Experiments that acquire NMR data for a compound over a range of pH and temperature values can help elucidate the structure. Atoms that are particularly sensitive to these changes will shift accordingly.

The structure of the amino acid hypusine was first determined with the help of NMR when it was initially isolated from bovine brain (Shiba et al., 1971). Two hypusine-containing dipeptides, α-(β-alanyl)-hypusine and α-(γ-aminobutyryl)-hypusine, as well as hypusine-containing eIF-5A peptide fragments, were analyzed by various NMR techniques in this dissertation. A discussion of their ^{1}H NMR spectra is presented below. The successful incorporation of deoxyhypusine, hypusine and ^{13}C-labeled hypusine reagents into eIF-5A (human) peptide fragments (Chapter 4) now provides the ability to study the 20 amino acid portion of the protein that surrounds the Lys_{50}-Hyp_{50} modification site. Both 1D and 2D ^{1}H and ^{13}C NMR analyses have been conducted on the eIF-5A peptide fragments (data are listed in Chapter 4). Detailed peak assignments are discussed below as well. In addition, several 2D NMR analyses have been conducted on the eIF-5A peptide fragments in order to more completely assign the NMR spectra and

provide an NMR fingerprint of this interesting region of the protein. To date, only crystal structures of eIF-5A have been reported and these structures do not contain the hypusine modification (Peat et al., 1998; Kim et al., 1998). These crystal structures clearly show that the hypusine modification site is positioned away from the main body of the protein, easily accessible to both solvent and additional proteins and enzymes (i.e. DOHS, DOHH). Acquiring NMR data of this post-translational modification region in solution phase may help to eventually elucidate the mechanisms by which hypusine imparts complete functionality to its parent protein, eIF-5A.

Analysis of Hypusine Dipeptides

The two hypusine dipeptides synthesized in this study, β-Ala-Hyp and GABA-Hyp, have been analyzed by routine 1D ^{1}H and ^{13}C NMR on a 300 MHz Varian NMR system (room temperature, pH ~2.0). Their chemical shifts are tabulated in Chapter 4. The chemical shifts for the amino acid hypusine have been reported elsewhere and were used to help assign the NMR spectra of the dipeptides (Bergeron et al., 1993). An assigned hypusine ^{1}H NMR spectrum is included in this dissertation for comparison to the dipeptides (Fig. 6-1). This section is devoted to a more detailed discussion of the ^{1}H NMR spectra for the dipeptides.

Results and discussion. Intuitively, the chemist should realize that the NMR spectra for these two dipeptides should be strikingly similar to each other as the structure of the compounds differs only by a $-CH_2$ group. Indeed, Figure 6-2 displays how the NMR spectra for the compounds are similar. First, both display a double doublet (δ = 4.06) and triple triplet (δ = 4.35) for the two chiral centers of the hypusine moiety, 2*S* and 9*R* respectively. These two sets of peaks correspond to a single proton each. It is

interesting to note that the alpha proton at C-2 of hypusine shifts from 3.75ppm to 4.35 ppm when hypusine is coupled to β-alanine or GABA. The splitting observed for the proton at C-9 (triple triplet) is the result of a proton on a chiral center being surrounded by two adjacent $-CH_2$ groups. The $N+1$ "splitting rule" predicts at least five peaks (Silverstein et al., 1991). Nine peaks are observed due to the chiral proton interacting with four adjacent protons having two possible spins each. Coupling constants (J) of 9.6 Hz and 3.2 Hz are observed for this pattern of peaks. Multiplets are observed for the $-CH_2$ groups adjacent to nitrogen atoms in the range of 3.00–3.23 ppm for both dipeptides. Integration of these peaks reveals eight hydrogens for each dipeptide as expected. In a similar manner, multiplets are observed for the alkyl groups in the range of 1.70–2.00 ppm. This multiplet integrates to six protons for the β-Ala-hypusine and eight protons for GABA-hypusine as expected. An additional multiplet is observed for the two protons at C-3 of the hypusine moiety in each peptide at 1.43–1.53 ppm.

Finally, there is perhaps one most noticeable feature to the NMR spectra of the dipeptides that helps to distinguish them from one another. In addition to the total integration of the spectra differing by two protons, there is a distinctive triplet at 2.75 ppm for β-Ala-hypusine and 2.45 ppm for GABA-hypusine. Integrals for both triplets indicate two protons with coupling constants of 6.7 Hz and 7.4 Hz respectively. These peaks correspond to the two protons adjacent to the carbonyl group on the β-alanyl and GABA residues of the peptides. Their chemical shifts are different due to the protons they are coupled to. In addition, this pattern of peaks differs in the manner in which it "leans" for each peptide. The triplet for these protons in the β-Ala-hypusine spectrum "lean" to the left because these protons are adjacent to a $-CH_2$ group (found downfield, or

to the left, in the multiplet observed near 3.0 ppm) next to a terminal free amine. When the second amino acid in the dipeptide is GABA, the chemical shift of this triplet moves to 2.45 ppm and leans upfield or to the right. This occurs because the distance between these protons and the free amine has increased due to the additional $-CH_2$ spacer on the GABA side chain. The chemical shift for this $-CH_2$ spacer is observed in the multiplet at 1.70 to 2.00 ppm and therefore the triplet at 2.45 ppm leans toward it. These characteristic leans and coupling help to distinguish these two hypusine-containing dipeptides via ^1H NMR.

Analysis of eIF-5A Peptide Fragments

It is important for eIF-5A peptides to be examined in their solution phase by NMR. Crystal structures obtained thus far on the immature protein indicate that the post-translational modification region protrudes from the main body of the protein, easily available for modification. The examination of these 20 amino acid fragments of the protein provides the basis for which to analyze the entire protein in its solution phase by NMR and compare it to its crystallized conformation. It is not completely unreasonable to expect the two states of the protein to show some difference. Analysis of the entire immature protein by NMR would be quite difficult without first obtaining detailed NMR data for specific portions of the protein such as the hypusine modification region discussed below. The availability of reliable hypusine reagents enables the possibility of the eventual complete synthesis of the eIF-5A protein. The NMR analysis of eIF-5A peptide fragments surrounding the hypusine modification site and containing the most highly conserved region of the eIF-5A protein in its three stages ($Lysine_{50}$, $Deoxyhypusine_{50}$, and $Hypusine_{50}$) is discussed below.

Initial 1D ^{1}H and ^{13}C analysis. The eIF-5A peptide fragments synthesized in this study have all been analyzed by routine 1D ^{1}H and ^{13}C NMR techniques on a 500 MHz Bruker Avance NMR system at the UF McKnight Brain Institute AMRIS Facility (chemical shift data have been reported in Chapter 4). Since these peptides were prepared synthetically and not isolated at random from a biological system, their sequences were already known. Initial NMR assignments were possible from the 1D spectra alone for these peptides based on their known sequence and tabulated chemical shift data for the 20 common amino acids (Bundi & Wüthrich, 1979; Krishna & Berliner, 1998; Reid et al., 1997; Dunn & Pennington, 1994). Typically, alpha protons of an amino acid in a peptide are located in the 3.90–5.0 ppm chemical shift range. This is due to the deshielding effects of neighboring nitrogen atoms and carbonyl groups in the peptide backbone. Amide protons that are not completely exchanged are observed in the 8.0–9.0 ppm range. Aromatic protons such as those found on the aromatic rings of histidine residues are located in the 7.0–8.0 ppm range. (It will be shown below how these chemical shifts can change as a result of pH). Methylene and methyl protons are typically located in the 0.6–2.5 range. Other aliphatic protons adjacent to a heteroatom (e.g. $-CH_2-NH_2$) are typically observed in the 3.0–3.5 ppm range.

As stated, many chemical shifts may be assigned simply from the 1D ^{1}H NMR spectra obtained for the eIF-5A compounds. However, as the number of amino acids in a peptide sequence increases, it becomes increasingly difficult to pinpoint the exact chemical shift of every proton of a particular residue. Solvent peaks may also interfere with an NMR spectrum as well. Initial experiments with the eIF-5A peptide fragments showed that analyses of these peptides at low temperature (7 °C) and acidic pH (~2.6 –

3.0) allowed for the observation of a maximum number of ^1H signals. A pH titration from acidic to neutral pH followed in order to gauge whether low pH was inhibiting any secondary structure.

Figures 6-3, 6-4, and 6-5 compare the 1D spectra of the 5-, 8-, 14-, and 20 (hypusine-containing) amino acid eIF-5A peptides. The number of overlapping peaks makes peak assignment difficult. However, assigning the chemical shifts of these peptides in a sequential manner beginning with the smallest peptide to the largest has eased this task greatly. Several amino acids within the peptide sequences stand out over the rest and act as markers in the NMR spectra. For example, the multiplets observed just below 1.0 ppm correspond to $-CH_3$ groups of Leu_{58} and Val_{56} in the 5mer spectrum for a total of 12 hydrogens. In the 20mer spectra, this multiplet also includes the $-CH_3$ groups of $Val_{41}(\gamma1,\gamma2)$ and $Ile_{40}(\gamma1, \delta1)$ for a total of 24 hydrogens. An additional marker is the doublet routinely observed near 1.4 ppm. These peaks correspond to the β-methyl group on the only alanine residue in the peptide sequence, Ala_{54}. A strong singlet peak can also be observed for the S-CH_3 of Met_{43} near 2.0 ppm. Sharp singlets are also seen for the aromatic hydrogens (δ, ε) of $HIS_{57,53,51}$ at 7.3 and 8.6 ppm respectively. The β-CH_2 groups for the histidine residues are routinely observed near 3.2 ppm with a broad peak for the ε-CH_2 groups of $LYS_{55,47,39}$ and $Hypusine_{50}$. Additional assignments are shown in the figures at the end of this chapter (Fig. 6-3,4,5). Analysis of the eIF-5A peptide fragments in a systematic manner, from smallest to largest, provides a model way to eventually synthesize and analyze the entire eIF-5A protein in solution phase by NMR.

pH Titration NMR Study of eIF-5A (Hypusine$_{50}$ and ^{13}C Hypusine) 20mers

The eIF-5A peptides were all initially analyzed at low temperature (7 °C) and acidic pH (2.6–3.0). This proved to be helpful with assignments of the various peptides as low temperature "locks out" any minor conformations of the peptide and low pH allows the observation of amide protons. The exchange of backbone amide protons with solvents like 90% H_2O/10% D_2O is reported to be at a minimum around pH 3.0 (Wüthrich, 1986). A pH titration study examining the eIF-5A Hyp$_{50}$ and ^{13}C-Hyp$_{50}$ containing 20mers was carried out to observe if pH had any observable effects on the NMR spectra of the compounds implicating secondary structure. Also, because the signal to noise ratio in NMR spectra increases as the square root of the number of scans taken, data was typically acquired overnight (between eight and 20 hours).

Experimental–Hyp50 eIF-5A 20mer pH titration. The 20 amino acid peptide fragment of eIF-5A containing Hyp$_{50}$ was subjected to a ^{1}H NMR pH titration study. NMR data were collected on a 500 MHz Bruker Avance Spectrometer at the UF McKnight Brain Institute AMRIS facility using the 5 mm TXI probe. The concentration of the sample was 5.0 mM (6.8 mg dissolved in 0.6 mL 90% H_2O / 10% D_2O). A coaxial insert tube containing TSP in D_2O was placed inside the NMR tube containing the peptide sample and served as the reference (set to 0.0 ppm). Data were obtained with the transmitter centered on the water peak (~4.8 to 5.0 ppm). The water peak was also reduced using presaturation protocols. One-dimensional ^{1}H NMR spectra (21 total) were obtained for the sample over the pH range 2.63–7.60 at roughly 0.20 to 0.30 pH increments. The pH (uncorrected for D_2O) was adjusted by adding μL amounts of 0.1 N or 0.01 N HCl or NaOH and measured with a Cole-Parmer electrode (Vernon Hills, IL)

that fit directly into the NMR tube containing the peptide sample. The sample temperature was maintained at 7 °C during data collection. Data were processed using the Bruker XWINNMR program resident to the spectrometer.

Experimental–^{13}C labeled Hypusine$_{50}$ eIF-5A 20mer pH titration. The 20 amino acid peptide fragment of eIF-5A containing the ^{13}C enhanced Hyp$_{50}$ residue was also subjected to a ^{13}C NMR pH titration study. NMR data were collected on a 500 MHz Bruker Avance Spectrometer at the UF McKnight Brain Institute AMRIS facility using the 5 mm BBO probe. The concentration of the sample was 6.3 mM (7.2 mg dissolved in 0.5 mL 90% H_2O / 10% D_2O). A coaxial insert tube containing TSP in D_2O was placed inside the NMR tube containing the peptide sample and served as the reference (set to 0.0 ppm). One-dimensional ^{13}C NMR spectra (eight total) were obtained for the sample over the pH range 2.95–7.10 at roughly 0.5 pH increments. ^{1}H spectra were also acquired just prior to each ^{13}C spectrum as described above for the nonlabeled 20mer (data is not shown as these spectra mimic that of the unlabeled 20mer Hyp$_{50}$). The pH (uncorrected for D_2O) was also adjusted in the same manner described above. The sample temperature was maintained at 27 °C during data collection. Data were processed using the Bruker XWINNMR program resident to the spectrometer.

Results–Hyp$_{50}$ eIF-5A 20mer pH titration. Stacked-plots of the data obtained for the ^{1}H NMR experiment of the Hyp$_{50}$ eIF-5A 20mer at 21 pH increments (7 °C) are shown with various expansions in Figures 6-6 and 6-7. As expected, several peaks in the NMR spectrum shift as a result of the pH change from 2.63 to 7.60. Most notably, the multiplet centered at 2.475 ppm for the $-CH_2$ (γ) of Glu$_{42}$ at low pH shifts upfield to 2.275 ppm at neutral pH (Δ = 0.2 ppm). The amide proton for glutamic acid is also

observed to shift upfield (Δ = 0.45 ppm). At low pH, the E_{42} residue is fully protonated. Protonation diminishes towards neutral pH, thus changing the electronic environment surrounding the amide proton and causing it to be less deshielded and shifted upfield.

In a like manner, the peaks corresponding to the aromatic hydrogens of the three histidine residues in the 20mer also shift upfield as the pH value increases. Interestingly, the –CH (δ) hydrogens are observed as two singlets (2:1 ratio, near 7.3 ppm) at low pH, three distinct singlets at pH 5.00, and then two singlets again (1:2 ratio, near 7.1 ppm) at neutral pH. The signals for the –CH (ε) hydrogens of the histidine residues are also observed to change from a strong, broad singlet at low pH (around 8.6–8.7 ppm) to a broad multiplet at pH 5.00. The signals are split into three singlets further upfield (around 7.80 and 7.95) at neutral pH (Fig. 6-7). This peak shift is also a direct result of pH change from acidic to neutral.

Signals for side-chain amines observed at low pH were exchanged into the solvent as pH was increased, making these signals no longer visible above pH 5.00. In a like manner, several of the signals for the amides were also eliminated from the spectra as pH increased above 5.00. Thus, analyzing the peptides at low pH provided the advantage of observing the most proton signals and allowed for a more complete NMR assignment of the compound.

Results–^{13}C Hyp$_{50}$ eIF-5A 20mer pH study. Figures 6-8, 6-9, and 6-10 display the stacked plots of the ^{13}C spectrum of the labeled peptide. It is clear from these spectra that a single carbon on the hypusine residue was successfully labeled (Fig. 6-8). The signal for the enhanced carbon is nearly 10 times the intensity of all the other carbon peaks in the spectrum (Fig. 6-10d). The spectra for the labeled compound were acquired

with the "decoupler on". When the decoupler is on, single peaks are observed for each carbon. The N+1 rule does not apply. When the decoupler is turned off, a coupled spectrum is acquired. These spectra show the spin-spin splitting between carbon-13 atoms and the protons that are directly attached to them. A coupled spectrum was acquired at pH 2.95 and 7 °C to verify the type of carbon on which the enhanced ^{13}C label was incorporated. Carbon-11 of the hypusine side chain is a $-CH_2$. With two hydrogen atoms, a coupled spectrum should reveal a triplet (n+1 = 2H + 1 = 3 peaks). This observation further verified the successful incorporation of the ^{13}C label into the hypusine molecule (Fig. 6-8b). The coupling constants for this triplet are J = 142.8/285.5 Hz. It is clearly seen that this particular peak is unaffected by the change in pH (Fig. 6-8a). This would suggest that the hypusine residue is not hydrogen bound to or masked by any other residue in this particular portion of the eIF-5A protein. This is not surprising since it is expected that the hypusine residue in the eIF-5A protein remains unobstructed allowing eIF-5A to be fully functional. The fact that this labeled peak is easily observed by NMR and unaffected over a wide pH range makes this compound a good candidate for use in future NMR studies attempting to assess the potential interaction of the hypusine residue of eIF-5A with other viral proteins like Rev and RRE.

As was the case in the 1D ^1H NMR spectra, certain peaks in addition to the prominent ^{13}C label on the hypusine reside act as markers to help assign the ^{13}C NMR spectra. Most noticeable are the two quartets for the two carbon atoms in trifluoroacetic acid (Fig. 6-9b,d). TFA salts of eIF-5A peptides are formed as a result of the HPLC purification process which utilizes 0.1% TFA as part of the mobile phase. As a result, a quartet for the -COOH carbon is centered around 166 ppm with J = 107.1/71.4/35.7 Hz.

A second quartet for the $-CF_3$ carbon is centered on 119 ppm with J = 583.0/291.4 Hz. Additional ^{13}C assignments for the ^{13}C Hyp50 eIF-5A 20mer are shown in Figures 6-9 and 6-10. Carbon signals belonging to the histidine residues were also easily identified. The remaining ^{13}C assignments were made in manner identical to that of assigning the 1D ^{1}H NMR spectra for the eIF-5A peptides—assignment of the smallest to longest peptide (data not shown, ^{13}C chemical shifts are listed in Chapter 4 for each peptide synthesized).

2D Analysis of eIF-5A Peptide Fragments

Despite the large amount of data obtained via one-dimensional NMR techniques, it was not possible to completely assign the eIF-5A peptide fragments solely with this data. Two-dimensional NMR techniques, with endearing acronyms, are routinely used to elucidate more precisely the chemical shifts of amino acid residues in compounds similar to eIF-5A peptide fragment. TOCSY or Total Correlation Spectroscopy, NOESY or Nuclear Overhauser Effect Spectroscopy, and HMQC, Heteronuclear Multiple Quantum Correlation spectroscopy are the names of the NMR techniques used in this dissertation for the analysis of hypusine-containing eIF-5A peptide fragments. A typical 2D spectrum appears like an "ink-blot" test to the casual observer–a symmetrical pattern of dots and blotches surrounding a central diagonal line. In these 2D experiments, the center diagonal line corresponds to the 1D spectrum of the peptide viewed "topographically" from above and the symmetrically placed cross-peaks on either side of the diagonal indicate the existence of interactions between two spins in the compound being analyzed.

TOCSY. In a TOCSY experiment, the cross peaks arise from all members of a coupled spin network. These interactions are also called "through-bond", scalar couplings (J, Hz). Through-bond couplings can allow the complete assignment of an

amino acid residue–from the amide proton to the alpha proton and all the way out along a side chain.

NOESY. In a NOESY experiment, the cross peaks arise as a direct result of dipolar couplings through space as opposed to through a bond network. The cross peaks indicate which protons are close to other protons in space (Krishna & Berliner, 1998). NOESY data is typically used to "walk" the peptide backbone from amide to amide and assign the peptide's sequence. This data can also be used to determine if a particular peptide or protein exhibits some sort of secondary structure. A NOESY cross peak could indicate the interaction of two protons on unique amino acids separated by several other amino acids in the peptide sequence. This would indicate that the peptide or protein is folded in some manner to allow these two protons to interact with one another. NOESY correlations occur at distances up to 5 Å. NOE intensity falls off very rapidly with increasing distance between proton pairs ($1/r^6$ where r is the distance between two nuclei) (Krishna & Berliner, 1998).

HMQC. Lastly, an HMQC experiment is an inverse chemical shift correlation experiment that is used to determine which protons of a molecule are bonded to which ^{13}C nuclei. This experiment is particularly useful if some of the ^{13}C nuclei are enhanced, such as via ^{13}C labeling of the hypusine residue in the 20 amino acid eIF-5A peptide fragment. This experiment would allow the pinpoint determination of the $-CH_2$ group in the $Hypusine_{50}$ sidechain in both the ^{1}H and ^{13}C spectra. This precise identification of the hypusine residue can be extremely advantageous for future studies examining the interactions of the eIF-5A peptide fragments, and particularly the hypusine residue, with other peptides and proteins such as Rev and RRE.

In order to help with the NMR assignments of the eIF-5A 20mers, several shorter peptide chains were synthesized and analyzed from shortest to longest. Peptides containing 5-, 8-, 14-, and 20 amino acid sequences were prepared and analyzed via 1D ^{1}H and ^{13}C NMR as discussed earlier and by various 2D experiments (TOCSY) discussed below. The NMR patterns observed in the spectra of the shorter peptide chains were easily followed and assigned in the larger peptide chains. As the peptide chain length increased, the newly added amino acids were observed and assigned as well. 2D TOCSY spectra for the various eIF-5A peptide sequences synthesized in Chapter 4 are described below. 2D NOESY data was acquired for the eIF-5A 20mers ($Lysine_{50}$, $Deoxyhypusine_{50}$, and $Hypusine_{50}$). The sequence of the Hyp_{50} 20mer was also "walked" using TOCSY and NOESY data acquired at 750 MHz and 7 °C. The eIF-5A 20mers containing $Lysine_{50}$ and ^{13}C-$Hypusine_{50}$ were also analyzed by HMQC NMR experiments in order to pinpoint the location of the protons on the labeled carbon. Experimental conditions and results are discussed below, followed by a discussion of the merits of the NMR data acquired in this dissertation and the potential this data provides for future study of hypusine and eIF-5A.

Experimental–2D TOCSY and NOESY NMR for eIF-5A peptides. The 5-, 8-, 14-, and 20 amino acid peptide fragments of eIF-5A with Lys_{50}, $DeoxyHyp_{50}$, and Hyp_{50} were subjected to a 2D TOCSY NMR analysis. The same samples prepared for the one-dimensional analyses were used again for the 2D experiments. All compounds were referenced to external TSP in D_2O. All samples were analyzed at 7 °C. For the eIF-5A 20mer with $Hypusine_{50}$, TOCSY data were also collected at three pH values (2.63, 5.00, and 7.60 at 7 °C, only data at 2.63 is shown below). An MLEV-17 mixing sequence was

applied for 70 msec with water presaturation for all TOCSY data acquisitions (Braunschweiler & Ernst, 1983; Bax & Davis, 1985). Two-dimensional NOESY data were collected for the eIF-5A 20mers at the same pH values and temperature listed for TOCSY acquisition. For NOESY data acquisitions, a 300 ms mixing time was used and water was suppressed with a 3-9-19 WATER-GATE sequence (Jeener et al., 1979; Wagner & Wüthrich, 1982). All spectra were acquired at 500 MHz over the course of 10-24 hours. In addition, TOCSY and NOESY data for the Hyp_{50} 20mer were also acquired at 750 MHz in order to gauge the advantage of higher magnetic field strength for the analysis of eIF-5A peptide fragments. Table 6-1 summarizes the various NMR experiments carried out on each peptide sample.

Experimental–2D HMQC NMR for Lys_{50} and ^{13}C Hyp_{50} eIF-5A 20mer. The 20 amino acid peptide fragment samples of eIF-5A containing Lys_{50} and ^{13}C Hyp_{50} were also analyzed via a 2D HMQC NMR under conditions listed above. Water suppression was not required since it is not observed in the carbon spectrum. The pulse sequence used for HMQC experiments is described elsewhere (Bax et al., 1983; Bax & Subramanian, 1986).

Results–2D TOCSY, NOESY and HMQC NMR for eIF-5A peptides. Results for the 2D NMR analyses for the various peptides synthesized in this study are best displayed pictorially. Figures 6-11 and 6-12 display matched expansions of the 5-, 8-, and 14-amino acid eIF-5A peptide fragments. Assignments for each amino acid residue are shown. For example, the cross peaks for the $-CH_3$ groups ($\gamma 1$, $\gamma 2$) of $Leucine_{58}$ are identified as L_{58} ($\gamma 1$, $\gamma 2$). Other amino acid peaks are identified by their traditional single letter abbreviation (e.g. **A** = **A**lanine, **K** = **Ly**sine, etc.), number in peptide sequence (e.g.

K_{50}), and proton type (e.g. –CH α) as well. For the purposes of space in the figures shown, $Deoxyhypusine_{50}$ and $Hypusine_{50}$ residue peaks are identified as $DHyp_{50}$ and Hyp_{50} respectively. Figure 6-13 displays matched expansions for the TOCSYs of the three 20mers with $Lysine_{50}$, $Deoxyhypusine_{50}$, and $Hypusine_{50}$. The change in the NMR spectra due to the addition of new protons from the deoxyhypusine and then hypusine sidechains is clearly seen. TOCSY data was also acquired at three pH values (2.63, 5.00, and 7.60) for the Hyp_{50} 20mer to complement the 1D ^{1}H NMR pH titration study done with this particular peptide. No significant peak shifts other than those described for the 1D ^{1}H NMR pH study of this compound were observed in the 2D spectra. Therefore, only spectra at pH 2.63 are shown. Also, significant signal for the amide protons was lost at the higher pH range due to solvent exchange (Fig. 6-6).

Figure 6-14 shows the data obtained for the HMQC analysis of eIF-5A Lys_{50} and ^{13}C Hyp_{50} 20mers. This side-by-side comparison clearly displays the ^{13}C-labeled peak for C-11 on the hypusine side chain.

Lastly, a case can be made for the analysis of the eIF-5A peptides at the highest magnetic field currently available for routine NMR sample analysis at the University of Florida McKnight Brain Institute–750 MHz. Figure 6-15 compares two regions of 1D ^{1}H NMR spectra for the Hyp_{50} 20mer at 500 and 750 MHz. It is clear to see that greater peak resolution is obtained at the higher magnetic field. In particular, several of the amide protons are resolved and the histidine α-protons are further removed from the pesky water peak. In addition, matched plots (side-by-side) of the TOCSY and NOESY data obtained for the eIF-5A Hyp_{50} 20mer are shown in Figure 6-16. Alone, the TOCSY data resembles that obtained at the lower field (Fig. 6-13c). However, when

superimposed with the NOESY data, the entire sequence of the Hyp_{50} 20mer is "walked" from end to end, Leu_{58} through Lys_{39} (TOC-NOE-TOC-NOE-etc.). The path for this sequence walk is shown in the α-proton region of the superimposed TOCSY/NOESY spectra where the typical distances between neighboring –NH protons are less than 5 Å (Fig. 6-17). Black peaks are TOCSY peaks (through-bond coupling) and gray peaks are NOESY peaks (through space couplings). This complete sequence assignment could not be made at pH 5.00 or 7.60 due to extensive exchange of the amide protons with the solvent. No NOESY interactions were observed between amino acids that were not immediately adjacent to one another in the peptide sequences at low pH. If this interaction occurred, it would have indicated some sort of folding or bending in the peptide sequence allowing non-adjacent residues to be less than 5Å apart. Therefore, secondary structure or lack thereof of these particular peptides cannot be determined under the conditions employed here. Chemical shifts obtained for the amino acids in these eIF-5A peptide fragments at low temperature (7 °C) and acidic pH (2.6–3.0) are consistent with random coil values.

Discussion

Clearly a vast amount of NMR data has been obtained in this study. Chemical shifts have been recorded and amino acids have been carefully and thoughtfully assigned. The advantages of high magnetic field strengths, enhanced atoms, and multiple dimension NMR acquisitions have been detailed. Taken alone, the acquisition of this data may not be completely fascinating and may be even considered routine in this "modern age" of NMR. However, the acquisition and effective compilation of this data

was imperative for future study of hypusine and eIF-5A. Several of the merits of this study are discussed below.

Recall that Peat et al. (1998) examined the crystal structures of eIF-5A from *Pyrobaculum aerophilium*. This version of eIF-5A was found to be a β-structure with two distinct domains and three separate hydrophobic cores (Peat et al., 1998). The hypusine modification site, Lys_{42} for this species (analogous to Lys_{50} for human), was observed at one end of the protein in a turn between β strands. The lysine residue was found to be freely solvent accessible and was therefore thought to be readily available for modification by deoxyhypusine synthase, DOHS—the enzyme responsible for the first step in the biosynthesis of hypusine on eIF-5A (Fig. 1-4b). The researchers concluded that the modification of lysine to hypusine would not significantly alter the conformation of the entire eIF-5A protein in any major way (Peat et al., 1998). A study by João et al. confirmed this and reported that 40% of the molecules of unmodified (non-hypusine containing) eIF-5A are present in a conformation similar to that of fully mature (hypusine-containing) protein (João et al., 1995).

As evidenced by NMR data obtained in this dissertation, the structures of the peptides containing the 20 amino acids of the highly conserved region of eIF-5A appeared to be unaffected by the incorporation of lysine, deoxyhypusine, or hypusine at position 50 of the peptide. Chemical shift data acquired for these eIF-5A peptide fragments agreed well with random coil values. Evidence for any secondary structure in these peptides was unobservable under the NMR conditions utilized in this dissertation. And, no evidence for any significant conformational change was observed for the hypusine containing peptide, and in particular the hypusine residue, over the range of pH

values tested (2.63–7.60). This evidence is consistent with the argument that hypusine, still protonated and cationic at physiological pH (as are its polyamine relatives), is at least in part responsible for eIF-5A's interaction with additional peptides, proteins, DNA, and/or RNA and thus important for the functionality of eIF-5A. The unique polycationic nature of the polyamines is thought to be a major reason why they exhibit a wide range of effects in nature, such as the stabilization of polyanionic molecules like DNA and RNA (Jänne et al., 1991; Igarashi et al., 1982). The polycationic nature of hypusine in eIF-5A may in part be responsible for hypusine-containing eIF-5A's role as a cellular cofactor of the HIV-1 Rev trans-activator protein (Bevec & Hauber, 1997).

A study by Kim's group also examined the crystal structure of eIF-5A from *Methanococcus jannashii* (Kim et al., 1998). Again, the most noticeable feature of the protein was the protruding finger comprised of the 12 highly conserved amino acids surrounding the hypusine modification site (S_{34}-K_{40}-K_{45}–analogous to S_{44}-K_{50}-K_{55} in the human eIF-5A sequence). (Recall that this was the region of focus for peptide syntheses in this dissertation). They concluded that this sequence of amino acids was critical for successful hypusine modification to occur on this protein. However, they did report that 9- and 16mer peptide sequences surrounding this modification site did not function well as substrates for DOHS (Kim et al., 1998). As will be discussed below, these highly conserved amino acids may still play a key role.

One reason these peptides may not have functioned well as substrates for DOHS is that they may lack significant secondary structure. NMR data obtained in this dissertation indicates that these peptides adhere to a random coil configuration at low temperature and acidic pH. A lack of secondary structure may potentially inhibit the

availability of the lysine residue to be effectively accessed by the DOHS enzyme. It is quite possible that the lysine residue can be modified to hypusine only when the peptide sequence is folded in such a manner so as to position the lysine residue away from the main body of the eIF-5A protein, as seen in the crystal structures described above. The synthetic availability of such peptides discussed in this dissertation now makes testing this hypothesis feasible.

Recall the sequence of the eIF-5A 20mers synthesized in this study (Fig. 3-8). It should be noted that of the 20 amino acids present, three are histidine residues ($His_{57,53,51}$). Peptide complexes with metal ions are well documented and the role of the imidazole moiety of histidine in the binding of metal ions has been observed in proteins and in both natural and synthetic peptides (Spiga et al., 2002). Knowing now from the NMR data obtained here that the incorporation of deoxyhypusine or hypusine into the eIF-5A 20mers may have little to no effect on the structure of the peptide and that pH change is negligible as well, it may be possible to induce and mimic the extended, protruding loop of eIF-5A via the addition of a non-paramagnetic metal ion such as Ni(II) or Cu(II). A non-paramagnetic metal ion is required if NMR studies are to be utilized to examine the effects of a metal ion on the conformation of the peptides. The avoidance of any paramagnetic metals in the presence of an NMR magnet is always strongly encouraged. Even small amounts of a metal such as Fe(III) in the NMR sample would make acquiring a useful spectrum difficult. If the addition of a metal ion to the peptide solution indeed caused a complex to form with the histidine residues, the peptide could very well bend in such a way as to allow the lysine residue to extend out, as in the crystal structures.

The NMR data obtained in this dissertation could be used as a basis for observing peptide structure that could be potentially induced via the addition of a metal ion to the eIF-5A peptide fragments in solution. TOCSY and NOESY data, which were unobservable for the 20mers at neutral pH due to extensive amide proton/solvent exchange, may become prominent with the addition of a metal ion and the induction of secondary structure, thus potentially bringing several protons of widely separated residues in to close proximity to one another (Spiga et al., 2002; Gaggelli et al., 2001). The comparison of solution NMR data obtained above for the eIF-5A 20mers with the potentially metallo-ion complexed peptides and previously observed crystal structures for eIF-5A may further elucidate the role of a metal complex requirement for the post-translational modification of lysine to hypusine on eIF-5A. Possibly, the metal ion (Fe^{3+}) at the active site of deoxyhypusine hydroxylase (DOHH) could be drawn in close to the eIF-5A intermediate by the histidine residues near the hypusine modification site (Csonga et al., 1996; Abbruzzese et al., 1991). The histidine residues may guide the metal ion in such a way as to position the enzyme to hydroxylate the deoxyhypusine residue. In addition, successful induction of secondary structure causing the lysine and deoxyhypusine residue to extend from the peptide may provide additional substrates for the isolation and purification of DOHS and DOHH.

In a similar manner, it may be possible to induce a loop structure in the peptide sequences synthesized by modifying the synthesis to include cysteine residues at each terminus of the peptide. After each peptide containing lysine, deoxyhypusine, or hypusine is synthesized on the resin via SPPS, a disulfide bridge can be induced between the cysteine residues (Grant, 1992). This would essentially "pin back" the amino- and

carboxy-termini of the peptides possibly allowing the Lys_{50}-Hyp_{50} modification site to protrude as observed in crystal structure studies. Alternatively, a cyclic structure might be obtained by modifying the synthesis to allow the Lys_{55} ε-amino group to couple to the Glu_{42} carboxy terminus. This would, in effect, also bring the ends of the peptide together and possibly allow the hypusine modification site greater access away from the main body of the peptide. In either case, the availability of detailed spectral data obtained in this dissertation provides a strong basis by which to observe future structural variants of the eIF-5A peptides by multiple dimension NMR techniques. If this forced conformational change is successful, additional studies may be employed to observed the interactions of linear hypusine-containing peptides versus cyclic hypusine containing peptides with other peptides such as Rev. The ability to easily incorporate a ^{13}C label into such peptides would also facilitate the observations of the hypusine residue with other biomolecules by NMR.

In summary, the synthesis of eIF-5A peptides of varying length and analysis of these compounds via 1D and 2D NMR has provided an NMR fingerprint by which to analyze larger hypusine-containing eIF-5A peptide fragments and eventually the entire, fully mature, hypusine-containing eIF-5A protein. Systematic synthesis of larger peptides and subsequent analysis by NMR with comparison to the data obtained here may allow the determination of the minimal peptide sequence required to observe chemical shift data that deviates from random coil values and therefore imply secondary structure. Nevertheless, the integrity of the hypusine reagents was established and the feasibility of utilizing such reagents in extended peptide syntheses was verified. Lastly, the ^{13}C label incorporated into the eIF-5A peptide is a powerful tool in itself as it allows the researcher

to easily observe the hypusine residue by NMR. It is possible that once the lysine residue is extended (i.e. hypusine formation), it is available to interact with additional peptides, proteins, and other biomolecules. This labeled peptide can now be used to observe the interactions of eIF-5A with DNA, RNA, other peptides, and viral proteins. As larger peptide fragments, and ultimately the entire eIF-5A protein, containing the set of hypusine reagents described here are prepared, it may be necessary to incorporate additional labels into the peptides. This can easily be done via the uniform isotopic labeling of a protein. ^{15}N-enhanced amino acids (amide), which are commercially available, can be incorporated into the SPPS techniques described earlier quite easily. NMR experiments called ^{15}N-^{1}H HSQC, Heteronuclear Single Quantum Coherence (similar to HMQC), can be used to further analyze these peptides and determine any secondary structure that might be present on larger eIF-5A peptide portions at various temperature and pH conditions. Therefore, the various NMR techniques employed in this dissertation have helped to characterize hypusine, its dipeptides, and the 20-residue portions of eIF-5A. Clearly, an important basis of NMR tools for further study of the area of the protein that undergoes this interesting post-translational modification has been firmly established.

Table 6-1. Summary of NMR Experiments on eIF-5A Peptide Fragments

Peptide	[ ] mM	^{1}H	^{13}C	TOCSY	NOESY	HMQC
5mer (A54 – L58) **(20)**[d]	7.7	✓	✓	✓	---	---
8mer (H51 – L58) **(21)**	7.5	✓	✓	✓	---	---
14mer, Lys_{50} (T45 – L58) **(22)**	5.9	✓	✓	✓	---	---
20mer, Lys_{50} (K39 – L58) **(23)**	4.9	✓	✓	✓	✓	✓
20mer; $DHyp_{50}$ (K39 – L58) **(24)**	4.0	✓	✓	✓	✓	---
20mer; Hyp_{50} (K39 – L58) **(25)**	5.0	✓ pH 2.63–7.60[a]	---	✓ pH 2.63, 5.00, 7.60[c]	✓ pH 2.63, 5.00, 7.60[c]	---
20mer, ^{13}C Hyp_{50} (K39 – L58) **(26)**	6.3	✓	✓ 7 °C & 27 °C pH 2.63 – 7.10[b]	---	---	✓
21mer, Hyp_{50} (C38 – L58) **(27)**	1.4	✓	---	---	---	---

Notes: Peptide sequences are shown in Fig. 3-7, 3-8. A "✓" indicates that a spectrum whose type is indicated in the column header was acquired for that particular peptide. A "---" indicates that a particular NMR experiment was not acquired. All NMR data were acquired at 7 °C and referenced to external TSP in D_2O

a) This sample was analyzed via an NMR pH titration experiment from pH 2.63-7.60 (21 pH values)
b) In addition to ^{13}C analysis at 7 °C, this sample was analyzed via an NMR pH titration experiment at 27 °C from pH 2.63-7.10 (8 pH values)
c) These samples were analyzed at the three pH values listed in the table
d) Indicates compound number for reference to Chapter 4

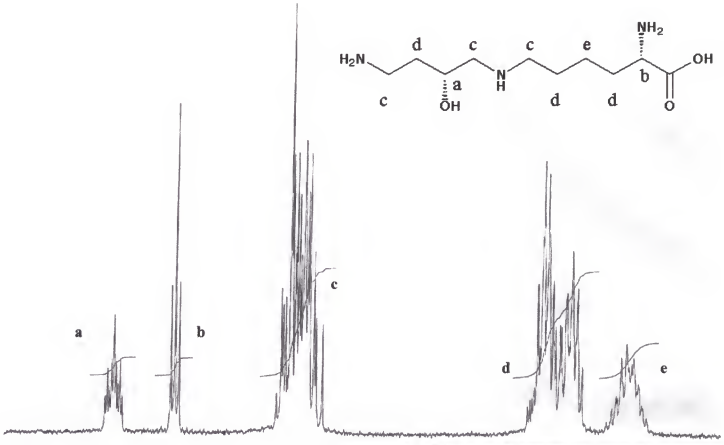


Figure 6-1. 1D ^{1}H NMR of 2S, 9R Hypusine (300 MHz)

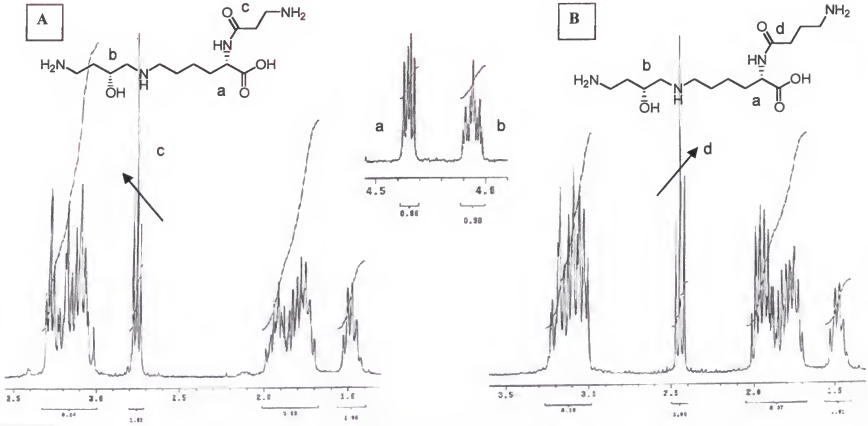


Figure 6-2. 1D ^{1}H NMR (300 MHz) of Hypusine Dipeptides–A) α-(β-alanyl)-Hypusine and B) α-(γ-aminobutyryl)-Hypusine

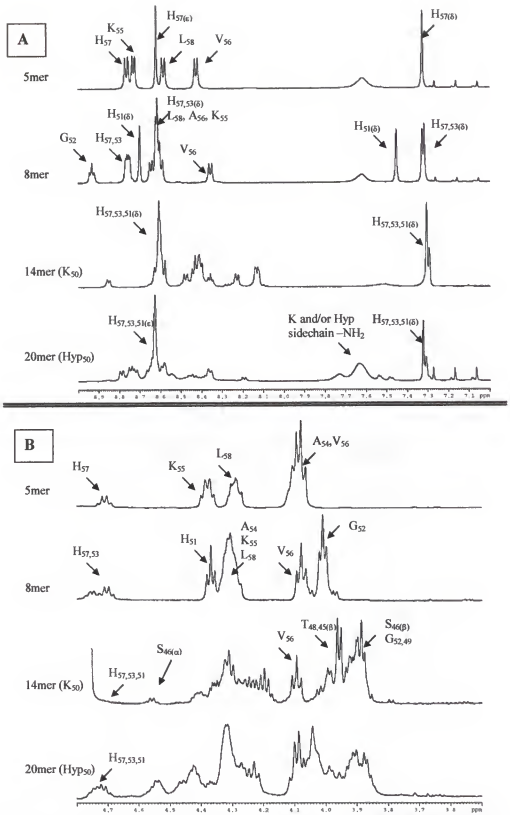


Figure 6-3. 1D ^{1}H NMR (500 MHz, 7 °C) of eIF-5A 5, 8, 14, and 20mer (Hyp_{50}) Peptides–A) Amide (-NH) and Histidine Aromatic Region (9.0–7.0 ppm) and B) α-^{1}H Region (4.8–3.5 ppm)

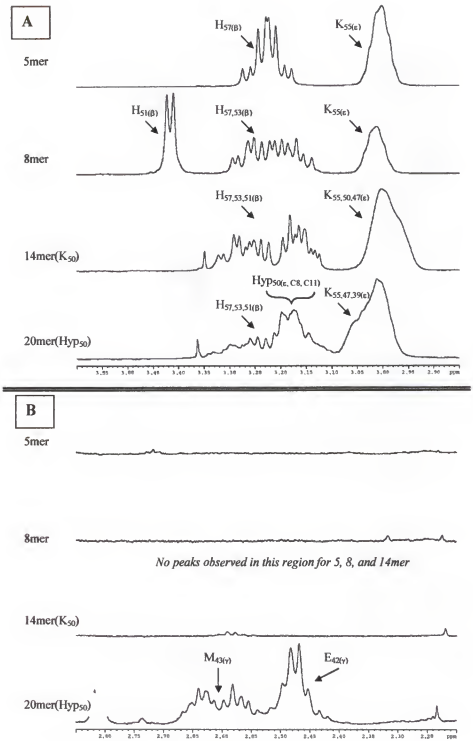


Figure 6-4. 1D ^{1}H NMR (500 MHz, 7 °C) of eIF-5A 5, 8, 14, and 20mer (Hyp_{50}) Peptides–A) His β-CH_2 and Lys ε-CH_2 region (3.60–2.80 ppm) and B) M_{43} and E_{42} –CH_2 γ (2.85–2.20 ppm)

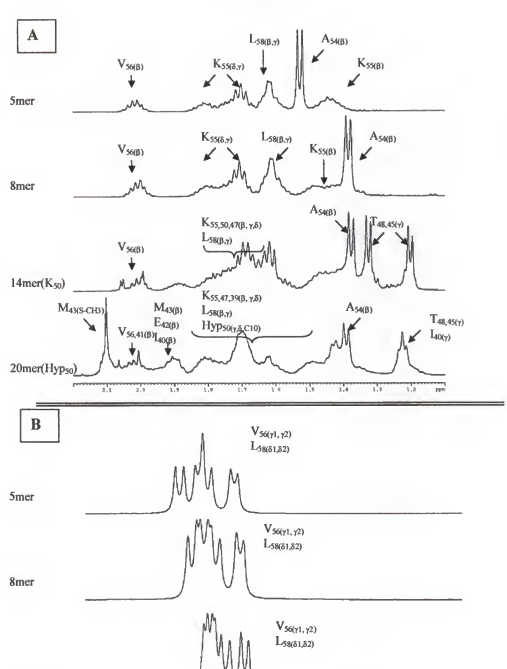


Figure 6-5. 1D ^{1}H NMR (500 MHz, 7 °C) of eIF-5A 5, 8, 14, and 20mer (Hyp_{50}) Peptides–A) Aliphatic Region (2.2–1.1 ppm) and B) Terminal Methyl Groups (1.1–0.5 ppm)

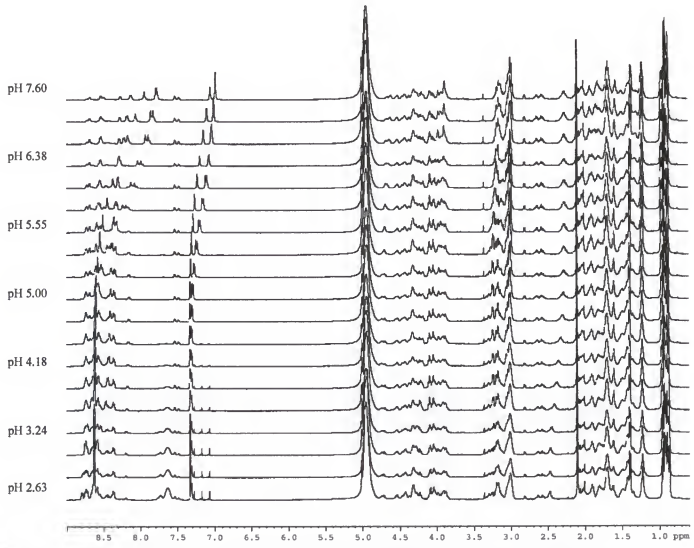


Figure 6-6. 1D ^{1}H NMR (500 MHz, 7 °C) of eIF-5A 20mer (Hyp_{50})–pH Titration from 2.63 to 7.60

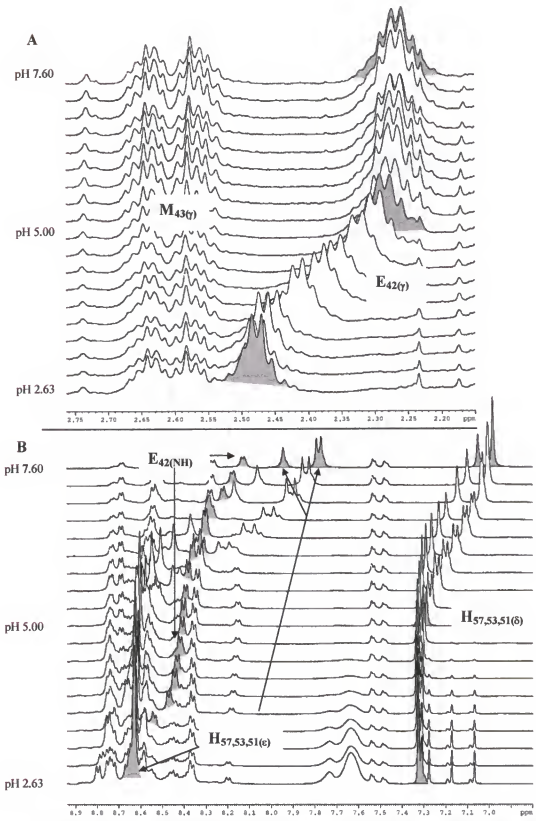


Figure 6-7. 1D ^{1}H NMR (500 MHz) of eIF-5A 20mer (Hyp_{50})–pH Titration Expansions A) 2.75–2.10 ppm and B) Amide/Aromatic Region 8.9–6.8

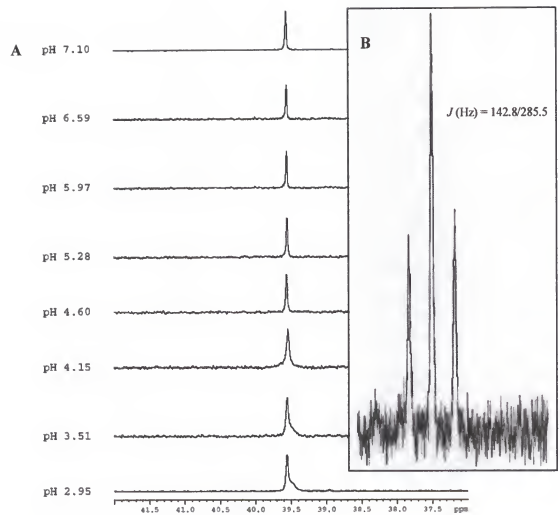


Figure 6-8. ^{13}C NMR (27 °C) of labeled Hyp_{50} -CH_2 on C11 of Hypusine Side Chain–A) pH Titration from 2.95–7.10 and B) Coupled ^{13}C Spectrum of C11, pH 2.95

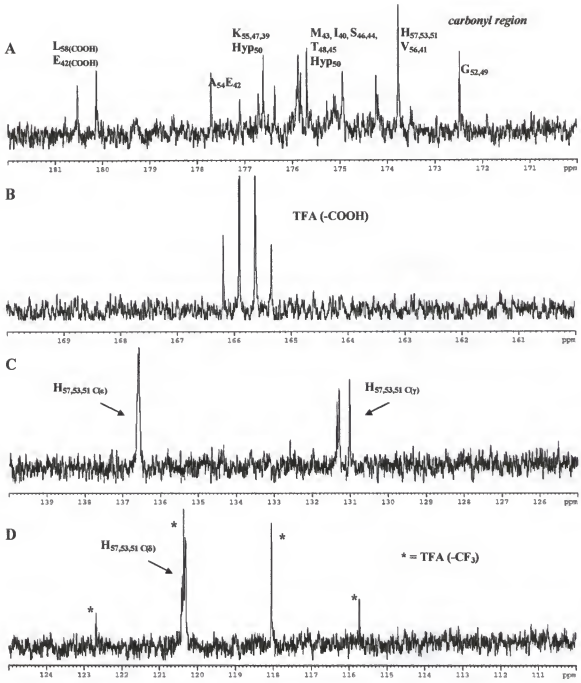


Figure 6-9. ^{13}C NMR Spectra Expansions of eIF-5A 20mer for ($^{13}CHyp_{50}$), A) 182–170 ppm, B) 170–160 ppm, C) 140–125ppm, D) 125–110ppm

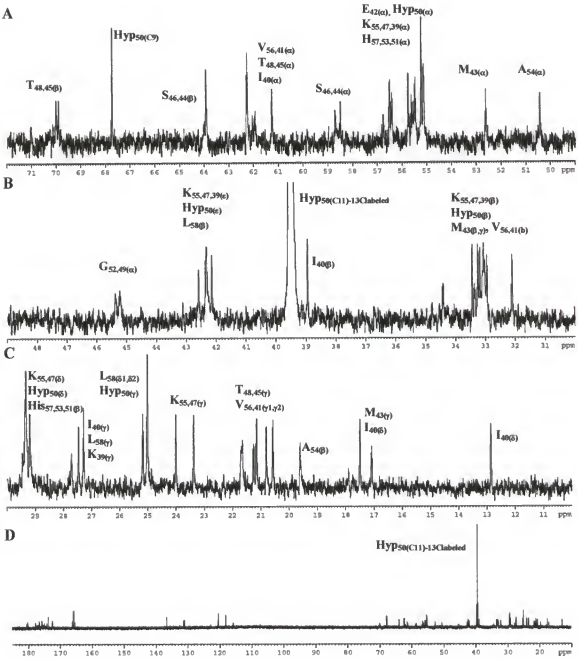


Figure 6-10. ^{13}C NMR Spectra Expansions for eIF-5A 20mer (Hyp_{50}), A) 72–49 ppm, B) 49–30 ppm, C) 30–10, D) 182–10 ppm

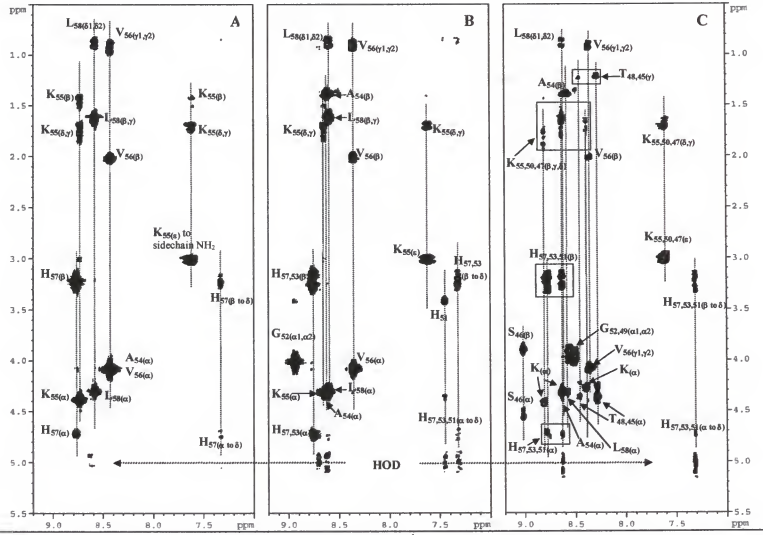


Figure 6-11. TOCSY NMR Spectra (500 MHz, 7 °C), Aliphatic and α-^{1}H to Amide Region–A) 5mer (L_{58}-A_{54}), B) 8mer (L_{58}-H_{51}), C) 14mer (L_{58}-K_{50}-T_{45})

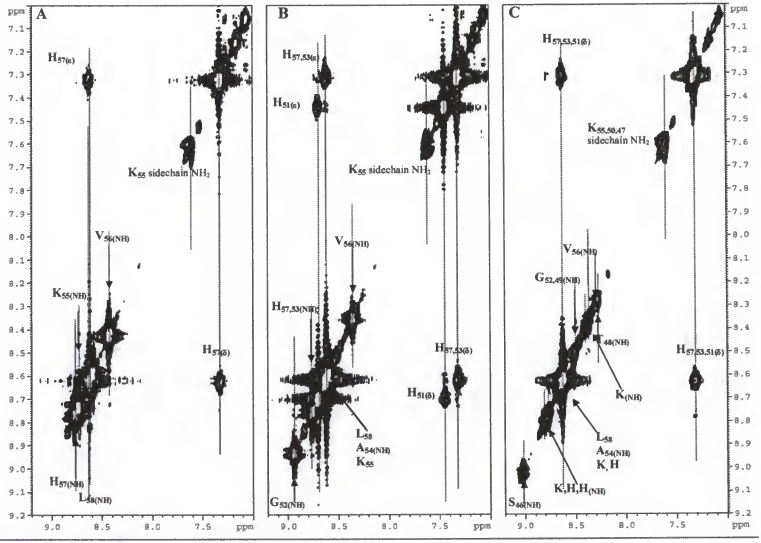


Figure 6-12. TOCSY NMR Spectra (500 MHz, 7 °C), Amide Region–A) 5mer (L_{58}-A_{54}), B) 8mer (L_{58}-H_{51}), C) 14mer (L_{58}-K_{50}-T_{45})

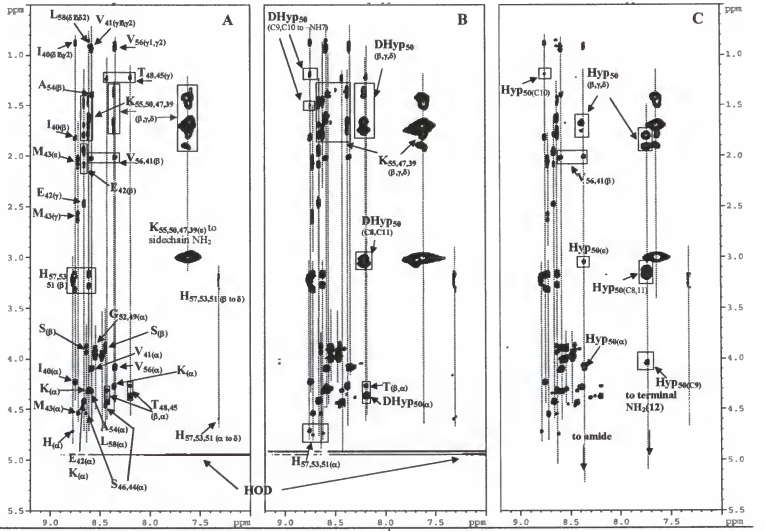


Figure 6-13. TOCSY NMR Spectra (500 MHz, 7 °C), Aliphatic and α-^{1}H to Amide Region–A) 20mer (K_{50}), B) 20mer ($DHyp_{50}$), C) 20mer (Hyp_{50})

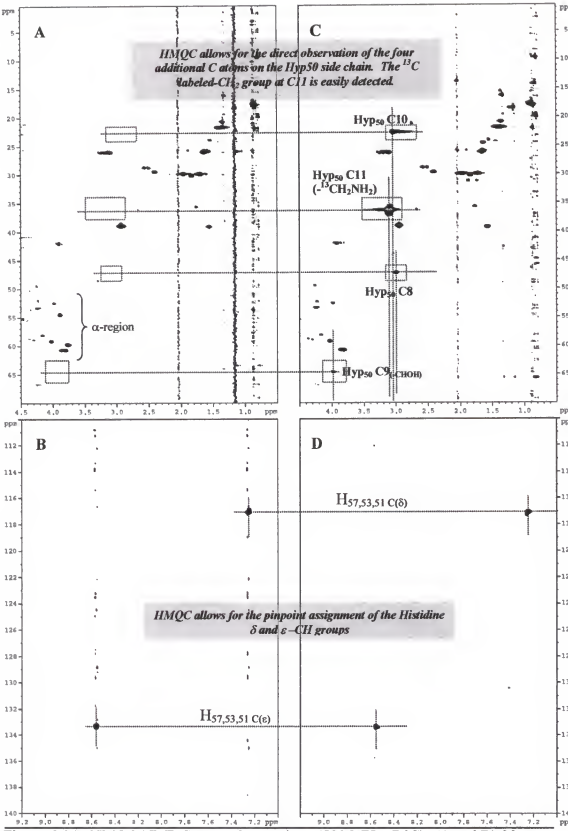


Figure 6-14. HMQC NMR Spectra Comparison (500 MHz, 7 °C)–A) and B) 20mer (Lys_{50}); C) and D) 20mer ($^{13}CHyp_{50}$)

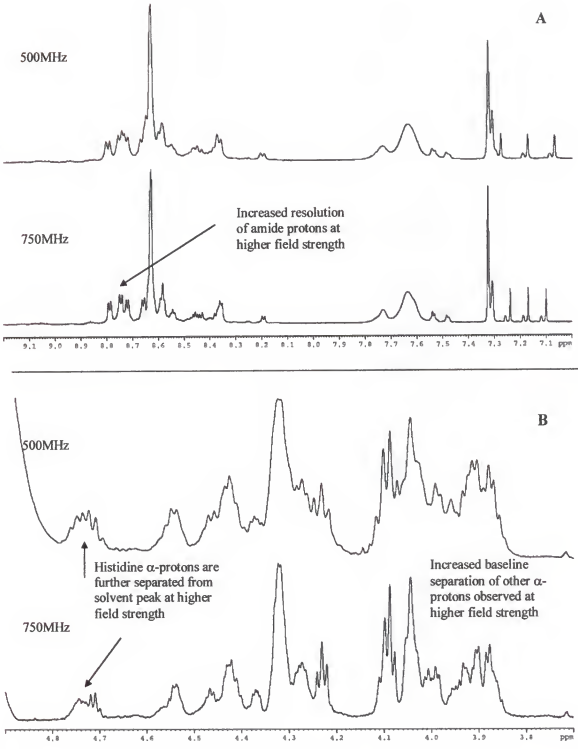


Figure 6-15. Comparison of 1D ^{1}H NMR Spectra for eIF-5A 20mer (Hyp_{50}) at 500 MHz and 750 MHz–A) Amide/Aromatic Region 9.2–7.2 and B) α-^{1}H Region 4.9–3.7

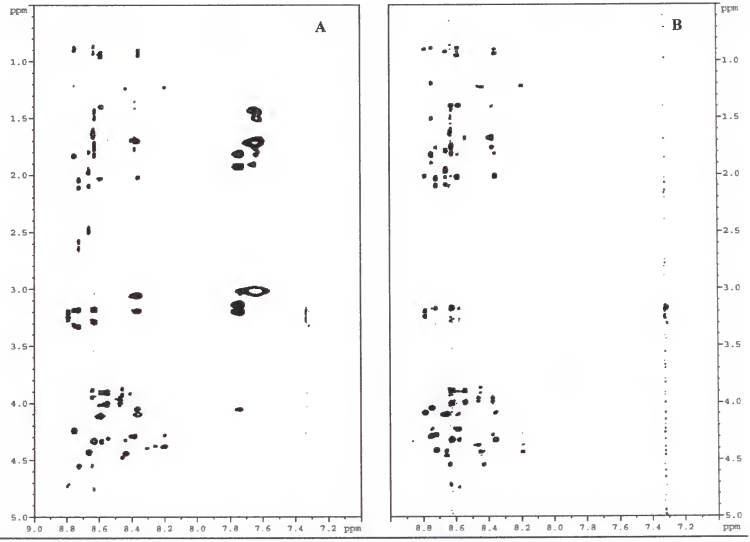
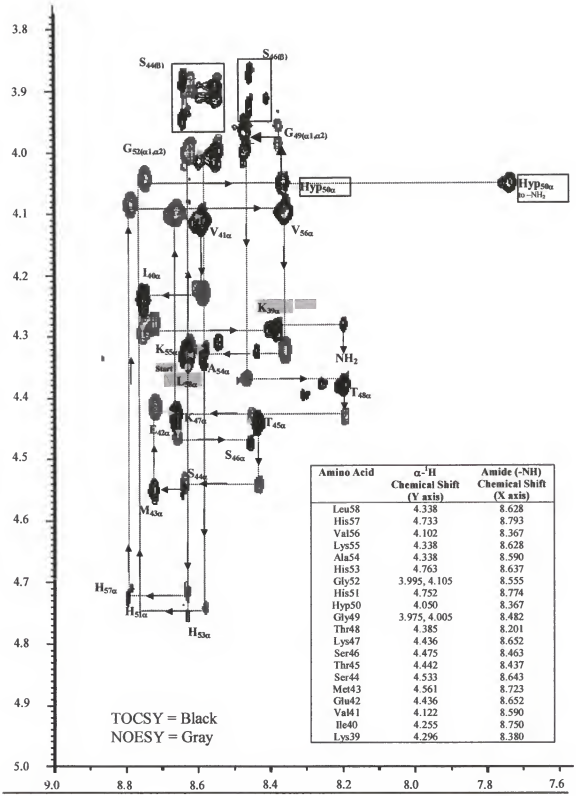


Figure 6-16. eIF-5A 20mer (Hyp_{50})–(A) TOCSY vs. (B) NOESY, 750 MHz, 7 °C



Amino Acid	α-^{1}H Chemical Shift (Y axis)	Amide (-NH) Chemical Shift (X axis)
Leu58	4.338	8.628
His57	4.733	8.793
Val56	4.102	8.367
Lys55	4.338	8.628
Ala54	4.338	8.590
His53	4.763	8.637
Gly52	3.995, 4.105	8.555
His51	4.752	8.774
Hyp50	4.050	8.367
Gly49	3.975, 4.005	8.482
Thr48	4.385	8.201
Lys47	4.436	8.652
Ser46	4.475	8.463
Thr45	4.442	8.437
Ser44	4.533	8.643
Met43	4.561	8.723
Glu42	4.436	8.652
Val41	4.122	8.590
Ile40	4.255	8.750
Lys39	4.296	8.380

Figure 6-17. Superimposed TOCSY and NOESY for eIF-5A 20mer with Hyp_{50} (750 MHz, 7°C)–Sequence Assignment from NMR Data–α-^{1}H to Amide Region

CHAPTER 7
SUMMARY AND CONCLUSIONS

Research to determine the exact functions of hypusine, its various peptides, and its parent protein, eIF-5A, has been hindered by the lack of appropriate scientific tools. Key to these studies is the development of reagents and standards and analytical methods that can be adapted to detect these compounds in various biological samples.

In this dissertation, the synthesis and analysis of hypusine dipeptides, ^{3}H-hypusine, and the incorporation of hypusine, deoxyhypusine, and ^{13}C labeled hypusine reagents into eIF-5A peptide fragments has been explored. A new tissue extraction method and both HPLC and HPLC-MS assays for the analysis of free hypusine and hypusine dipeptides from rat brain have also been developed. Finally, the characterization of eIF-5A peptide fragments containing lysine, deoxyhypusine, hypusine, and ^{13}C labeled hypusine (position 50 in human eIF-5A) by both 1D and 2D high field homonuclear and heteronuclear experiments has been completed.

The successful synthesis of the hypusine dipeptides, α-(β-alanyl-hypusine) and α-(γ-aminobutyryl)-hypusine, provided an efficient means to access these previously unavailable standards. The standards were then used for the development of an HPLC and HPLC-MS analytical assays for these compounds along with their parent amino acid, hypusine. Extracts of rat brain homogenates were analyzed for these compounds via precolumn OPA/NAC derivatization on HPLC and HPLC-MS systems. "Suspect peaks" were observed to coelute with standard spikes of hypusine and GABA-hypusine by HPLC analysis. HPLC-MS analysis confirmed only the presence of hypusine in tissue

extracts. HPLC-MS analysis also confirmed that OPA/NAC precolumn derivatization produces a di-derivatized compound.

The preparation of a novel ^{3}H-labeled hypusine compound offers a great advantage to studying this unique amino acid further in biological systems. In addition to serving as a tracer compound for the development of efficient extraction methodologies as described in this dissertation, ^{3}H-hypusine could also act as a tracer in metabolic studies. Small amounts of the labeled compound may be spiked into a portion of the diet of a small animal such as a rat or mouse. Or, a small amount of the compound could be injected directly into the laboratory animal. Parallel to other metabolic experiments, urine and fecal samples could be collected over the course of several days after which the animal is euthanized. Various tissues such as the intestine, liver, kidneys, and brain would then be harvested. The urine, fecal, and tissue samples could then be extracted and analyzed via techniques such as the HPLC assay described earlier only utilizing a radiolabeled detection unit. The peaks detected by the radiolabeled detector could then be collected and further analyzed to determine which organs the compound has passed through. And, if any have been formed, hypusine metabolites could also be detected. This method would also allow the researcher to determine if hypusine localizes in a particular tissue or tissues, as ^{3}H-labeled hypusine and/or its metabolites would be observed in certain sample extracts and not in others. This knowledge may help to further define if there is a functions or role of this enigmatic amino acid as a free polyamine.

In addition, the HPLC analytical methods described in this dissertation allow for the detection of hypusine dipeptides as well. Placing the ^{3}H-hypusine into a biological

system and then analyzing various tissues can help to determine if free hypusine is being incorporated into hypusine-dipeptides like α-(β-alanyl)-hypusine and α-(γ-aminobutyryl)-hypusine. Recall that α-(β-alanyl)-hypusine and α-(γ-aminobutyryl)-hypusine peptide sequences are not located in the 154 amino acid human eIF-5A protein (or any other species examined to date). These dipeptides were not detected in the extracts of rat brain homogenates under the conditions employed in this study. This radioactive reagent may help to identify a novel enzymes involved in the coupling of free hypusine to other amino acids such as β-alanine and γ-aminobutyric acid. Placing a mass spectrometer in tandem with the HPLC and radiolabeled detector could also enhance the detection of hypusine, its dipeptides and possible metabolites present in urine, feces, and various tissues.

The development of a hypusine reagent that can be incorporated into eIF-5A peptide fragments can also serve as a valuable tool to study hypusine, its functions, and its relationship to eIF-5A. The synthesis and analysis of these eIF-5A peptide fragments may help to provide a more detailed understanding of the amino acid region surrounding the hypusine modification site in the protein. Additionally, by characterizing these eIF-5A peptide fragments and hypusine dipeptides by NMR, "fingerprint" NMR spectra were obtained and can now be used for comparison and reference in future experiments.

The successful incorporation of deoxyhypusine and hypusine reagents into the eIF-5A protein sequence, with reasonable yields, provides the basis for the eventual manual synthesis of the entire eIF-5A protein by solid phase peptide synthesis techniques. One of the main difficulties in studying this unique protein and the post-translation event that it undergoes is the lack of suitable substrates to analyze the

enzymes that affect this change. Deoxyhypusine hydroxylase does not recognize the free amino acid deoxyhypusine. To date, entire eIF-5A sequences have been cloned but only with the lysine residue at the hypusine modification site. In this dissertation, the reagents incorporated into peptides of 20 amino acid residues provide the basis for the synthesis of much larger eIF-5A peptide sequences, and perhaps the entire eIF-5A sequences containing lysine, deoxyhypusine, and hypusine at position 50. The synthesis of such proteins would provide substrates to facilitate the complete characterization of both the hydroxylase and synthase enzymes that synthesize hypusine on eIF-5A.

The possibility of inducing structure into these relatively small eIF-5A peptide fragments via the incorporation of a metal-ion complex and/or disulfide bridge may provide additional substrates for the isolation and characterization of DOHS and DOHH. The inactivity of these enzymes on linear peptides similar to those synthesized in this dissertation may be due in part to the hindered access of these enzymes to the hypusine modification site. The successful preparation and characterization of these eIF-5A peptides easily leads to the preparation and characterization of their cyclical counterparts. The systematic synthesis of longer eIF-5A peptides and analysis and comparison to the NMR data reported here would allow the precise determination of the minimum amino acid sequence required for secondary structure and/or substrate function to occur.

Also, as ^{3}H-hypusine is easily detected via a radioactivity detector, a ^{13}C-hypusine containing compound can easily be detected via ^{13}C-NMR techniques. Direct injection of these reagents into *in vitro* or *in vivo* biological systems may help to further confirm or disprove previous researchers discoveries of free hypusine and hypusine dipeptide pools in the mammalian brain (Sano et al., 1986, 1987; Ueno et al., 1991). Under the

conditions employed in this study, only the presence of free hypusine in rat brain extracts could be confirmed by HPLC-MS. The dipeptides were not detected. Therefore, the hypusine reagents developed in Bergeron's laboratories and described here can be used to study this unique amino acid and its parent protein in an effort to demonstrate their metabolic functions and sources. Once the successful preparation of cyclical versions of the eIF-5A peptides has been established and its structure has been compared to that previously obtained from crystal formations of the eIF-5A protein, this labeled reagent could also be incorporated. In this manner, the potential interaction of the hypusine residue with various biomolecules such as Rev may be readily observed via NMR.

The successful incorporation of hypusine into an eIF-5A peptide fragment with a terminal cysteine residue (Cys_{38}) is yet another powerful tool. Not only can cysteine residues potentially affect cyclization of the peptide via disulfide bond formation, but monoclonal antibodies may also be developed for these larger hypusine-containing peptides, as was done previously for a hexapeptide containing hypusine (Bergeron et al., 1998a). These antibodies could aid in the elucidation of the function of hypusine containing peptides and determine if they are involved in nuclear import and/or export. The entire eIF-5A hypusine containing protein has been implicated in the viral replication of HIV as a cofactor for the nuclear export of the virus in conjunction with Rev-RRE (Bevec et al., 1996). Mutant studies where hypusine modification on eIF-5A was prevented have shown that Rev-RRE nuclear export and thus viral replication were inhibited (Bevec & Hauber, 1997). The potential for the synthesis of larger hypusine-containing peptides and developing antibodies to them may be quite useful in localizing intracellular hypusine-containing peptides and analogues. The NMR "fingerprint" data

along with the labeled peptide can be used as a basis for the future observation of the interactions of eIF-5A and hypusine with DNA, RNA, other peptides, and viral proteins. This data can greatly assist future researchers in studying eIF-5A and eventually elucidating its exact function(s) and role(s).

Clearly, the additional tools developed and described in this study should allow future researchers to further define the roles of the amino acid, hypusine, and its parent protein, Eukaryotic Initiation Factor 5A. Increased knowledge of this amino acid and protein and their relationships to other biological systems, facilitated by the experiments in this dissertation, may someday lead to a novel therapeutic outcome.

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BIOGRAPHICAL SKETCH

Matthew Joseph Della Vecchia was born in Bronxville, New York, on September 23, 1975. He lived in Mount Vernon, New York, with his family until they moved to Palm Coast, Florida, in June 1989. Matthew graduated from Flagler Palm Coast High School with honors in May 1993. He was accepted to the University of Florida and received his Bachelor of Science degree in chemistry with honors in December 1996. Matthew immediately began his graduate school studies toward a doctoral degree in January 1997 under the direction of Dr. Raymond J. Bergeron in the Department of Medicinal Chemistry at the University of Florida. Matthew was married to his college sweetheart, Dr. Stephanie A. Stancil, Pharm.D. (UF College of Pharmacy, Class of 1999), on May 30, 1999. He received his Ph.D. in pharmaceutical sciences-medicinal chemistry in August 2002.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Raymond J. Bergeron, Chairman
Graduate Research Professor of
Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Margaret O. James
Professor of Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John H. Perrin
Professor of Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard R. Streiff
Professor of Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Charles A. Allen
Professor of Biochemistry and Molecular
Biology

This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2002

Dean, College of Pharmacy

Dean, Graduate School